THE ROLE OF P53/MIR34/MET PATHWAY IN EPITHELIAL OVARIAN CANCER PATHOGENESIS

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The miR-34 family is directly transactivated by tumor suppressor p53, which is frequently mutated in the majority of human cancers, including epithelial ovarian cancer (EOC). MET receptor protein tyrosine kinase is one of the common targets of miR-34 family. MET activation is frequently implicated in increased motility and invasion of cancer cells and is associated with poor prognosis of EOC patients. However, it remains uncertain if dysregulation of p53/miR-34/MET signaling pathway plays an important role in EOC pathogenesis.

During our studies, we have found frequent downregulation of miR-34 in human EOC. Reduced expression of miR-34 family was associated with *p53* mutations, promoter methylation and copy number variations. At the same time, levels of miR-34a and MET expression were inversely correlated in EOC specimens. miR-34 reconstitution in p53 deficient ovarian cancer cells resulted in reduced proliferation, motility and invasion, the latter of which was dependent on MET expression.

Consistently, we were able to find an increase of MET expression in the mouse ovarian surface epithelium after conditional *p53* inactivation, resulting in increased cell motility and invasion. Concomitant inactivation of *Met* and *p53* abrogated this feature, suggesting that MET activation was responsible for *p53* control of cell motility and invasion. In addition to miR-34-dependent regulation of MET, we also identified miR-34-independent mechanism, in which p53 inhibits SP1 binding to *MET* promoter. miR-34-dependent and -independent mechanisms are

not functional in p53 absence, while mutant p53 proteins retain partial *MET* promoter suppression. Accordingly, MET expression, cell motility and invasion were particularly high in p53 null cells, as compared to cells expressing mutant p53 protein. This finding may provide a mechanistic explanation to reports of poorer prognosis of patients with p53 null EOC as compared to those expressing mutant p53.

In order to further explore the role of miR-34 family in development and carcinogenesis, mice carrying conventional and conditional targeted mutations of *mir-34* genes were generated. *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ mice are viable and exhibit phenotypes not typical for those associated with p53 null background. Specifically, mice do not develop early onset lymphomas but manifest hypoplasia of the white pulp in the spleen, accelerated thymic aplasia, focal hyperplasia of Purkinje neurons and ulceration of the forestomach, indicating miR-34 importance for a number of biological processes. Loss of *mir-34* resulted in increased cell proliferation, similar to that after p53 loss. However, unlike p53 deficiency, lack of *mir-34* did not increase induced pluripotent stem (iPS) cell reprogramming efficiency, suggesting that p53 may affect reprogramming by other mechanisms, in addition to increased proliferation. Our observations that *mir-34b/c* deficiency caused radioresistant phenotype after 15 Gy ionizing radiation also indicate that miR-34 role in DNA damage response is distinct from that of p53,

Taken together, we have shown that p53/miR-34/MET signaling pathway plays an important role in human EOC. Furthermore, we have identified miR-34-dependent and miR-34-independent mechanisms of MET regulation by p53 and determined that existence of such mechanisms may explain uneven patient prognosis as a function of *p53* mutation type. Finally, generation of *mir-34* deficient mice allowed us to determine new biological functions of miR-34 during development and DNA damage response, laying ground for future studies aimed to reveal miR-34 roles in development, DNA damage response and carcinogenesis.

BIOGRAPHICAL SKETCH

Chang-Il Hwang was born on October 9th, 1977, in Seoul, South Korea. Dedicated to a career in Biomedical Sciences, Chang-Il obtained his Doctoral degree in Veterinary Medicine from Seoul National University. Having more interests in biomedical research, he pursued master degree in Biochemistry and Molecular Biology in College of Medicine, Seoul National University. As a part of military service required for all Korean men, he worked at Biomedlab Co., a bio-company manufacturing diagnostic microarrays for two and half years. In 2006, Chang-Il came to USA and started to pursue his Ph.D. degree at Cornell University, Ithaca, NY, where he joined the laboratory of Dr. Alexander Nikitin to study the role of p53 and miR-34 in mouse models of human cancer.

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LIST OF ABBREVIATIONS

AML	Acute myeloid leukemia
AP	Alkaline phosphatase
BAC	Bacterial artificial chromosome
ChIP	Chromatin immunoprecipitation
CLL	Chronic lymphocytic leukemia
DIG	Digoxigenin
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EOC	Epithelial ovarian cancer
GI tract	Gastrointestinal tract
HGF	Hepatocyte growth factor
iPS	Induced pluripotent stem
IR	Ionizing radiation
LIF	Leukemia inhibitory factor
LNA	Locked nucleic acid
MEF	Mouse embryonic fibroblast
miRNA	microRNA
mitA	Mithramycin A
MSP	Methylation specific PCR
NSCLC	Non-small-cell lung carcinoma
OSE	Ovarian surface epithelium
p53 RE	p53 response element
qRT-PCR	Quantitative reverse transcription polymerase chain reaction

RISC	RNA induced silencing complex
rtTA	tetracycline reverse transcriptional activator
TKO	Triple knock-out
TTF	Tail-tip fibroblast

CHAPTER 1

INTRODUCTION

1.1 p53 and ovarian cancer

The *p53* gene was first identified in 1979 as a target of the SV40 oncogenic virus (Linzer and Levine, 1979) and initially proposed as an oncogene (Eliyahu et al., 1984; Jenkins et al., 1984; Parada et al., 1984). Later, p53 has been shown to be an important tumor suppressor gene that functions as a key transcriptional regulator (Levine and Oren, 2009). As a transcription factor, p53 is able to bind to a consensus response element (p53 RE, two tandem copies of the sequence PuPuPuC(A/T)(A/T)GpyPyPy separated by a 0-13 bp) and transactivate target genes, such as *p21, Mdm2, Puma, Bax* and *Gadd45*. In addition, p53 is capable of repressing a large number of genes by either DNA binding or protein-protein interaction (described in Chapter 1.3), although molecular mechanisms of such repression remain poorly understood (Ho and Benchimol, 2003; Rinn and Huarte, 2011). Due to large number of its target genes, p53 plays a pivotal role in coordinating the cellular responses to a broad range of cellular process including cell cycle arrest, DNA repair, apoptosis, senescence and angiogenesis (Menendez et al., 2009).

p53 mutations are the most common genetic alteration in human cancers (Levine and Oren, 2009). *p53* mutations frequently occur in DNA binding domain, resulting in loss of its function as a transcriptional activator. In addition to loss of function by rendering p53 inactive, some mutations result in proteins with new transcriptional properties, so called gain of function mutants (Reviewed in (Oren and Rotter, 2010; Vousden and Prives, 2009)). In some cancer

types, gain of function mutations have been reported to be associated with more aggressive and metastatic characteristics as compared to those in cancers with p53 null phenotype (Brosh and Rotter, 2009). However, it is controversial whether certain gain-of-function mutants predict poorer prognosis than p53 null in all human cancers (Soussi and Beroud, 2001). Indeed, as compared to malignancies expressing mutant p53 protein, p53 null neoplasms have been shown to be associated with poorest prognosis in lung, breast and ovarian cancers (Hashimoto et al., 1999; Lai et al., 2004; Olivier et al., 2006; Rose et al., 2003; Rossner et al., 2009; Shahin et al., 2000; Sood et al., 1999).

Ovarian cancer is the sixth most frequent female cancer worldwide, with 200,000 new cases diagnosed every year, and the most lethal of all gynecological cancers (Jemal et al., 2011; Jemal et al., 2010). Epithelial ovarian cancer (EOC) is the most common type of ovarian cancer, and is further classified into 4 different major groups (serous, mucinous, endometrioid and clear cell carcinoma) based on tumor cell morphology (Cho and Shih, 2009). Among these, high grade serous EOC is the most common type, and is characterized by high frequency of *p53* mutation, fast progression, and the poorest prognosis (Cho and Shih, 2009; Choi et al., 2008; Corney et al., 2008). The majority of EOC is believed to originate from the ovarian surface epithelium (OSE), a single layer of cuboidal cells coating the ovary (Auersperg et al., 2001; Nikitin et al., 2004).

During ageing OSE frequently form inclusion cysts which are considered to be precursor lesions. Consistent with this possibility, overexpression of p53 is often found in such cysts (Okamura and Katabuchi, 2001). Supporting that *p53* mutations are an early event during epithelial ovarian carcinogenesis they have been also detected in early stage high grade serous adenocarcinomas, as well as in adjacent dysplastic epithelium of prophylactically removed ovaries from patients with BRCA1 heterozygosity (Werness et al., 2000). In agreement with critical roles of p53 in EOC pathogenesis, OSE-specific inactivation of *p53*, particularly

combined with *Rb* gene, results in malignant neoplasms closely resembling human high grade serous adenocarcinoma of the ovary according to their pathology, biological behavior and gene expression signatures (Flesken-Nikitin et al., 2003).

Notably *p53* mutation is mainly detected in high grade serous adenocarcinoma of the ovary, but is rarely present in low grade serous carcinoma. Since the latter neoplasm has frequent *K-ras* mutations but no *p53* mutations, it is generally accepted that high and low grade carcinomas have distinct pathogenesis (Cho and Shih, 2009). At the same time, *Pten* mutations are present more frequently in endometrioid adenocarcinoma of the ovary. Consistent with association of specific genetic alterations particular tumor types, OSE-specific *Pten* inactivation combined with either mutated *K-Ras* expression or aberrant Wnt/beta-catenin signaling results in endometrioid carcinoma in genetically modified mice (Dinulescu et al., 2005; Wu et al., 2007).

In addition to the OSE cell of origin, some EOC derive from the epithelium of the fallopian tube fimbria and other derivatives of the Mullerian tract based on recent morphologic, immunohistochemical and molecular genetic studies (Dubeau, 2008; Kurman and Shih Ie, 2011; Lee et al., 2007; Medeiros et al., 2006). A hypothesis unifying EOC origin from OSE and fallopian epithelium has been also proposed based on following observations (1) the OSE and fimbrial epithelium have connection both developmentally and anatomically, (2) several differentiation markers are overlapped between two epithelia, particularly near their anatomic boundary, and (3) there are similar examples of transitional epithelia prone to neoplastic progression such as the squamo-columnar junction of the uterine cervix and the esophageal-gastric junction (Auersperg, 2011; Auersperg et al., 2008). Unfortunately, no convincing experimental support of this hypothesis has been reported so far and the origin of EOC from cell types other than the OSE remains largely speculative.

The need for understanding pathogenesis of the ovarian cancer is particularly urgent because over 70% of ovarian cancer patients are diagnosed in advanced stage and shows poor survival rate (Jemal et al., 2010) mainly due to asymptomatic and rapid EOC progression. At the same time, patients diagnosed with EOC confined to the ovary (stage I) have significant 5 years survival ranging from 60% to 90%, similar to other common cancers. As discussed below, recent discovery of p53/miR-34 network as well as better understanding of p53 transrepression mechanisms has opened an exciting opportunity for development of better diagnostic, therapeutic and preventive approaches cancer in general and EOC in particular.

1.2 miR-34 family in cancer and therapeutic promises

miRNA is a family of small non-coding RNAs which comprise 22~24 nucleotide lengths. Primary transcripts of miRNAs are transcribed mostly by RNA polymerase II, and some by RNA polymerase III. This primary transcript is called pri-miRNA. Pri-miRNA is processed by Drosha, RNase III endonuclease in the nucleus, resulting in ~70 nt double stranded RNA molecules called pre-miRNA. Subsequently pre-miRNA is transported into cytoplasm by exportin-5, and further processed into ~22 nt RNA duplex by another RNase III endonuclease, Dicer. A mature form of miRNA is loaded into RNA induced silencing complex (RISC), and bind to 3'-UTR of target genes resulting in mRNA cleavage or translational inhibition (Bartel, 2004; Huntzinger and Izaurralde, 2011; Krol et al., 2010). Over 60% of mammalian mRNAs are predicted targets of miRNAs (Friedman et al., 2009). Depending on its target genes, effect of miRNA could be oncogenic or tumor suppressive. miRNAs were initially linked to tumorigenesis due to their proximity to chromosomal breakpoints (Calin et al., 2004b) and their dysregulated expression levels in many malignancies (Calin et al., 2004a; Lu et al., 2005). During recent years, the

involvement of miRNAs in cancer pathogenesis has become increasingly obvious (Corney and Nikitin, 2008; Garzon et al., 2010).

One of the most intensively studied miRNAs is miR-34 family. miR-34 family is encoded by *mir-34a* gene located on human chromosome 1 and *miR-34b* and *mir-34c* genes located on human chromosome 11 (chromosomes 4 and 9, respectively, in mouse). miR-34a is transcribed alone, while miR-34b and miR-34b/c share a common primary transcript as a cluster. The first evidence for potential involvement of miR-34 family in cancer has arisen from reports of a minimal deleted region 11q23-q24-D containing *mir-34b/c* in lung and breast cancers (Calin et al., 2004b), and 1p37 containing *mir-34a*, frequently deleted in high-risk neuroblastoma (Cole et al., 2008; Welch et al., 2007). In 2007 several groups, including our laboratory, independently reported that miR-34 family is a direct target of p53 transactivation (Bommer et al., 2007; Chang et al., 2007; Corney et al., 2007; He et al., 2007; Tarasov et al., 2007).

Consistent with miR-34 transactivation by p53, frequent downregulation of miR-34 expression has been reported in ovarian cancer (Dahiya et al., 2008; Eitan et al., 2009; Iorio et al., 2007; Kuo et al., 2009; Zhang et al., 2008), non-small-cell lung carcinoma (Gallardo et al., 2009; Landi et al., 2010; Mudduluru et al., 2011), leukemia (Chim et al., 2010; Mraz et al., 2009; Zenz et al., 2009), neuroblastoma (Cole et al., 2008; Welch et al., 2007), colorectal carcinoma (Toyota et al., 2008), oral carcinoma (Scapoli et al., 2011), head and neck carcinoma (Lujambio et al., 2008), gastric cancer (Suzuki et al., 2010) and pleural mesothelioma (Kubo et al., 2011). This downregulation was associated with *p53* mutations, changes in expression of Myc, FoxP1 and C/EBPα expression, *mir-34* epigenetic inactivation or copy number variations (summarized in Table 1.1). Although p53 is a critical regulator to induce miR-34 family expression, a number of evidences support that there are more regulators of miR-34a and miR-34b/c such as c-Myc, C/EBPα, ELK1, p38 MAPK/MK2 and K-Ras (Cannell et al., 2010; Chang et al., 2008; Christoffersen et al., 2010; Kent et al., 2010; Pulikkan et al., 2010).

Table 1.1 Clinical significance of miR-34 family downregulation in human cancers

Cancer	Subtype	miR-34 member	Mechanism	Clinical significance	Reference
Ovarian cancer		miR-34c ↓		ND*	(Dahiya et al., 2008)
	Serous	miR-34c ↓		ND	(Iorio et al., 2007)
		miR-34a ↓		Accelerated cancer progression	(Eitan et al., 2009)
	EOC	miR-34a and b ↓		Accelerated cancer progression	(Zhang et al., 2008)
	Low grade EOC	*mir-34a* hemizygous deletion	Deletion	ND	(Kuo et al., 2009)
	EOC	miR-34a ↓	*p53* mutation, promoter methylation, and copy number variations	ND	Chapter 2 (Corney et al., 2010)
	EOC	miR-34 b/c ↓	*p53* mutation and promoter methylation	Accelerated cancer progression	Chapter 2 (Corney et al., 2010)
Lung cancer		miR-34a and c ↓		Poor prognosis	(Landi et al., 2010)
	NSCLC	miR-34a ↓		Increased relapse rate	(Gallardo et al., 2009)
	NSCLC	miR-34a ↓	Promoter methylation	ND	(Mudduluru et al., 2011)
	Stage I NSCLC	miR-34b/c ↓	Promoter methylation	Increased recurrence and poor survival	(Wang et al., 2011)
Leukemia	CLL	miR-34a ↓	*p53* mutation	ND	(Mraz et al., 2009)
	CLL	miR-34a ↓		Increased refractoriness to chemotherpay	(Zenz et al., 2009)
	High grade B cell lymphoma	miR-34a ↓	Myc and FoxP1 overexpression	ND	(Craig et al., 2011)
	AML	miR-34a ↓	*C/EBPα* mutation	ND	(Pulikkan et al., 2010)
Neuroblastoma		*mir-34a* deletion	Deletion	ND	(Cole et al., 2008)
		mir-34a deletion	Deletion	ND	(Welch et al., 2007)
Colorectal cancer		miR-34b/c ↓	Promoter methylation	ND	(Toyota et al., 2008)
Oral carcinoma		miR-34a ↓		ND	(Scapoli et al., 2011)
Pleural mesothelioma		miR-34a and b/c ↓	Promoter methylation	ND	(Kubo et al., 2011)
Gastric cancer		miR-34b/c ↓	Promoter methylation	ND	(Suzuki et al., 2010)

- NSCLC ; non-small-cell lung carcinoma, CLL; chronic lymphocytic leukemia, AML; acute myeloid leukemia
- * ND, not determined

Target of miRNAs can be computationally predicted by base paring between seed sequence of miRNAs and target gene 3'-UTR sequence (Bartel, 2004). However, a perfect seed match by itself has been shown to be a poor predictor for miRNA regulation due to the large number of random occurrences of any given hexamer in 3'UTRs. Perfect seed complementarity is neither necessary nor sufficient for miRNA regulation. Due to imperfect computational algorithms and lack of knowledge of detailed mechanism, current target prediction algorithms predict too many false-positive target genes (up to 66%), even though miRNAs regulate relatively small number of proteins at modest levels (Chi et al., 2009). Therefore miRNA-target gene relationship should be experimentally validated. Since miR-34 has large number of target genes like any other miRNAs and its effects are known to regulate proliferation, cell cycle, senescence, cell motility and invasion, miR-34 family is an attractive drug target for therapeutic purpose. Since miR-34 expression is reduced in many cancers, re-introduction of miR-34 into cancer tissue could provide a therapeutic benefit by restoring regulation of target genes. For example, miR-34a delivery has been shown to have tumor suppressive role in lung cancer models (Trang et al., 2011; Wiggins et al., 2010), pancreatic cancer (Pramanik et al., 2011) and neuroblastoma cells (Tivnan et al., 2011). Replacement strategies require delivery vehicles for delivery of double-stranded miRNA mimics. Due to intensive efforts to deliver siRNA prior to discovery of miRNAs, miRNAs seems ready to deliver systemically *in vivo* (Davis et al., 2010). In spite of similarity between miRNA and siRNA therapeutics, miRNA therapeutics have several advantages over siRNA approach, such as *in vivo* stability, highly RNA promoter-compatibility and no overt toxicity (Bader et al., 2010; Wang et al., 2009a).

1.3 Transcriptional repression by p53

While the mechanism of how p53 transactivates the promoter of target genes is well understood, details of transrepression by p53 remain less clear. Unlike transactivation which involves DNA binding of p53 onto the well-defined p53 RE, transrepression can occur through either DNA binding or protein-protein interaction (Ho and Benchimol, 2003). Unfortunately, DNA binding consensus sequence of p53, which is required for p53 binding on promoter regions, does not determine if p53 binding is associated with transactivation or transrepression. Furthermore, there is a novel p53 binding site that is oriented in an alternating head-to-tail formation instead of the head-to-head arrangement of the canonical p53 RE for transcriptional suppression (Johnson et al., 2005). Examples of DNA binding-dependent and DNA binding-independent modes of transcriptional repression by p53 are summarized in Table 1.2.

1.3.1 DNA binding-dependent, via canonical binding site mechanism

Approximately 15% of the p53 REs are associated with transcriptional repression (Riley et al., 2008). In this mode of repression, p53 DNA binding is required for transcriptional repression, and p53 REs are found in the promoter region. Although detailed mechanism still remains elusive, p53 may achieve transcriptional repression by two different DNA binding-dependent mechanisms. First, p53 is bound to p53 REs of promoter region, and subsequently recruits chromatin remodeling complexes. For example, in the *Survivin* promoter, upon DNA damage activated p53 is recruited in p53 RE of *Survivin* promoter, resulting in the recruitment of HDAC1, DNMT1 and histone methyltransferase G9a along with histone H3K9me2 (Esteve et al., 2007). In addition, Esteve et al. observed CpG island methylation in this region after p53 induction (Esteve et al., 2005). These results suggest that both DNA and histone methylation along with repressive complexes recruited by p53 mediates Survivin gene repression. Second, p53 compe-

Table 1.2 DNA binding-dependent and DNA binding-independent modes of transcriptional repression by p53 and their examples

Class	Mechanism		Gene	Interacting or competing partner	Reference
DNA binding-dependent	Canonical Head-to-head orientation PuPuPuC(A/T) (A/T)GpyPyPy	Chromating remodeling	Survivin		(Esteve et al., 2007)
			PKD1		(van Bodegom et al., 2010; Van Bodegom et al., 2006)
			HSP90AB1/Hsp90b		(Zhang et al., 2004)
		Compete with other transcription factor	miR-17-92	TBP	(Yan et al., 2009)
			POLD1	Sp1	(Li and Lee, 2001)
			CDC25B	NF-Y, Sp1	(Dalvai et al., 2011)
			Mn Superoxide dismutase	Sp1	(Dhar et al., 2008)
			dUTPase	Sp1	(Wilson et al., 2008)
	Noncanonical Head-to-tail orientation PuPuPu(A/T) PuPuPu(A/T)		MDR1		(Johnson et al., 2005)
			CD44		(Godar et al., 2008)
DNA binding-independent	Interaction with basal transcriptional machinery		Rb		(Shiio et al., 1992)
	Inhibit other transcription factor binding		Beta-amyloid precursor protein	Sp1	(Cuesta et al., 2009)
			MCL-1	Sp1	(Pietrzak and Puzianowska-Kuznicka, 2008)
			S100A6	Sp1, NF-kB	(Kroliczak et al., 2008)
			hTERT	Sp1	(Okawa et al., 2007)
			MGMT	Sp1	(Bocangel et al., 2009)
			HIV-LTR	Sp1	(Bargonetti et al., 1997)

tes with other transcription factor bindings. In this case, p53 RE is commonly located nearby other transcription factor binding sites, by which p53 binding prevents from other transcription factor bindings such as Sp1, NF-Y and TBP. For example, in the case of POLD1, MN Superoxide dismutase and dUTPase promoter, Sp1 binding site is located nearby p53 RE (Dhar et al., 2006; Li and Lee, 2001; Wilson et al., 2008). Gel shift assay and chromatin immunoprecipitation assay showed that p53 expression reduced Sp1 DNA binding onto the promoter region, suggesting that p53 occupancy in p53 RE competes with Sp1 DNA binding. Similar types of competition with TBP and NF-Y were also found in the promoter regions of miR-17-92 cluster and Cdc25b respectively (Dalvai et al., 2011; Yan et al., 2009).

1.3.2 DNA binding-dependent, via non-canonical binding site mechanism

Recently, non-canonical p53 RE is first identified in the *MDR1* gene (Johnson et al., 2005). While two copies of the sequence PuPuPuC(A/T) arranged head-to-head and separated by 0-13 base pairs are original p53 REs, the head-to-tail orientation highly similar to p53 RE was found in *MDR1* promoter, and p53 was able to bind this promoter region and suppressed *MDR1* promoter activity. Johnson et al. showed that when they replaced head-to-tail to head-to-head orientation *MDR1* promoter suppression was reversed (Johnson et al., 2005). Consistent with this observation, Godar et al. also found a non-canonical binding site in *CD44* promoter region, showing that only wild-type p53 was able to bind and repress *CD44* promoter activity not DNA binding mutant p53 (Godar et al., 2008).

1.3.3 DNA binding-independent mechanism

DNA binding-independent transrepression can be achieved through p53 interactions with other proteins, such as Sp1, NF-Y, NF-kB, TATA-binding proteins or basal transcriptional machinery. p53 has been shown to suppress Rb transcription through inhibition of the basal promoter activity already some time ago (Shiio et al., 1992). However, the detailed mechanisms how p53 suppresses promoter activities without p53 DNA binding remain only marginally explored. It has been reported that in DNA binding-independent mode p53 mutants still retain interaction with other transcription factors. This is of particular importance because p53 point mutations within DNA binding domain are among the most frequent in cancer (Oren and Rotter, 2010).

One of the common examples of p53 binding partner is Sp1, involved in regulation of several gene promoters, such as S100A6, MCL-1, hTERT, MGMT, HIV-LTR and beta-amyloid precursor protein (Bargonetti et al., 1997; Bocangel et al., 2009; Cuesta et al., 2009; Kroliczak et al., 2008; Pietrzak and Puzianowska-Kuznicka, 2008; Xu et al., 2000). In addition, DNA binding domain of Sp1 is known to interact with C-terminal of p53 (Koutsodontis et al., 2005), suggesting that p53-Sp1 interaction might affect the ability of Sp1 DNA binding. Consistent with this possibility, *in vitro* gel shift assay and chromatin immunoprecipitation experiments showed that p53 was able to reduce Sp1 DNA binding on its binding site of promoter regions in these genes (Bargonetti et al., 1997; Bocangel et al., 2009; Cuesta et al., 2009; Kroliczak et al., 2008; Pietrzak and Puzianowska-Kuznicka, 2008; Xu et al., 2000).

Notably, in both p53 DNA binding-dependent and -independent mechanism, Sp1 is involved in p53-mediated transcriptional repression. It is intriguing that genome-wide approach to identify p53 REs also indicated that potential Sp1-binding sequences were highly enriched in the vicinity of p53-binding sites (Smeenk et al., 2008). However, detailed mechanisms such as whether p53 activates or repress through p53 DNA promoter binding and whether p53 would

affect Sp1 DNA binding should be empirically tested and confirmed. Since presence of cofactor sites and the p53-RE occlusion by nucleosomes or other proteins may affect the role of p53 and make p53 RE as a nonfunctional, it should be interpreted in a cell-context dependent manner (Riley et al., 2008).

1.4 New p53 role as regulator of cell motility and invasion

Metastasis is responsible for most of cancer-associated mortality. It has been thought that a cancer cell from a primary tumor undergoes sequential steps : intravasation, extravasation and colonization (Chaffer and Weinberg, 2011). For the initiation of metastatic process, first of all cancer cells should acquire the ability to migrate and invade. As Hanahan and Weinberg recently revised hallmarks of cancer : (1) sustaining proliferative signaling, (2) evading growth suppressors, (3) avoiding immune destruction, (4) enabling replicative immortality, (5) tumor promoting inflammation, (6) inducing angiogenesis, (7) genome instability, (8) resisting cell death, (9) deregulating cellular energetics, and (10) activating invasion and metastasis (Hanahan and Weinberg, 2011), tissue invasion is one of the important hallmark of cancer. On the other hand, some hallmarks of cancer such as sustaining proliferative signaling, evading growth suppressors, enabling replicative immortality, angiogenesis and resisting cell death could also be characteristics of benign tumors (Lazebnik, 2010). Therefore, invading ability of cells might be the most important characteristic of cancer malignancies.

p53 has been thought to suppress carcinogenesis by regulating proliferation, apoptosis, senescence, angiogenesis and DNA repair (Zilfou and Lowe, 2009). However, the role of wild-type p53 in cell motility and invasion has not been studied in depth. It has been observed that wild-type p53 expression inhibits cell migration along with morphological alterations and actin

cytoskeleton changes (Alexandrova et al., 2000). Although detailed molecular mechanism remains elusive, it has been proposed that p53 regulates Rho family of small GTPase signaling, thereby controlling actin cytoskeletal organization and migration (Gadea et al., 2007; Gadea et al., 2002; Gadea et al., 2004). Members of this family include Rac, Cdc42 and Rho, controlling actin dynamics and cytoskeletal changes. However, a question whether this signaling represents the sole mechanism by p53 loss results in altered migration remains unanswered (reviewed in (Muller et al., 2011; Roger et al., 2006)). In addition, miR-34, a downstream target of p53 is known to downregulate Met, a receptor protein tyrosine kinase, which has been found to be overexpressed in a broad variety of human malignancies and is implicated in cell motility, invasion and metastasis (Birchmeier et al., 2003; He et al., 2007). While it is possible that p53 indirectly downregulates MET expression through miR-34, *Met* promoter has been shown to be activated by p53 (Seol et al., 1999). However, no studies directly linking miR-34 and/or p53 to regulation of MET-dependent motility has been reported so far.

At the same time, it has been reported that some gain-of-function p53 mutants, but not wild-type p53, may drive cell motility and invasion by promoting integrin recycling (Muller et al., 2009), by forming a complex with Smad to oppose p63-mediated control of putative metastasis suppressors Sharp-1 and cyclin G2 (Adorno et al., 2009) or by stabilizing the invasion facilitateor Slug (Wang et al., 2009b). Thus, it remains unclear whether wild-type p53 plays an important role in regulation of cell motility and invasion.

1.5 iPS cells reprogramming, p53 and miR-34

Utilizing stem cells for therapeutic purposes has been of interest due to its ability to self-renew and differentiate into several lineages of cell types. However, ethical issues related to obtaining

and use of human embryonic stem cells and limited capacity of self-renewal and differentiation of adult stem cells create critical hurdles for their therapeutic use. One of promising resolutions is to utilize induced pluripotent stem (iPS) cells. iPS cells have been generated from somatic cells by introducing four factors (Oct-4, Klf-4, Sox-2 and c-Myc) by Yamanaka group (Takahashi and Yamanaka, 2006). Since iPS cells are highly similar to embryonic stem cells and also it is possible to generate patient-specific iPS cells, potential for therapeutic applications of such cells is very high, albeit the safety issue on teratoma formation of iPS cells and the difference in epigenetic status has to be solved. Since some of Yamanaka's factors, particularly c-Myc and Klf4 are known oncogenes, numerous efforts to reduce the number of required factors and facilitate reprogramming efficiency were undertaken (Reviewed in (Wu and Hochedlinger, 2011)). It was shown that c-Myc improves iPS cell reprogramming process by facilitating cell proliferation but can be dispensable for reprogramming process (Wernig et al., 2008). More importantly, Wernig et al. showed that c-Myc-free iPS-derived chimera did not have enhanced tumor formation. In addition, in order to facilitate reprogramming and reduce the potential risk of virally-induced tumor formation, a number of approaches based on virus-free or vector-free reprogramming has been introduced (Kaji et al., 2009; Kim et al., 2009; Okita et al., 2008; Soldner et al., 2009; Stadtfeld et al., 2008; Yu et al., 2009). Moreover, iPS cells have been derived at increased efficiencies from easily accessible human cell types suitable for disease modeling, drug screening and potentially cell therapy in the future (Aasen et al., 2008; Haase et al., 2009; Kunisato et al., 2011; Loh et al., 2009; Lowry et al., 2008). By now, a number of additional iPS cell factors, such as Nanog, Lin28, Tbx3, ESRRB, UTF1, Sall4 as well as miR-291-3p, miR-294 and miR-295 have been reported to enhance reprogramming efficiency (Reviewed in (Judson et al., 2009; Okita and Yamanaka, 2010). Most recently, miR-302 and miR-372 have been shown to dramatically promote reprogramming efficiency (Subramanyam et al., 2011), and surprisingly even a single miRNA, miR-302 was able to reprogram human hair follicle cells to iPS cells (Lin et al., 2011).

One of the interesting factors able to improve iPS cell reprogramming efficiency is p53. In 2009, number of reports showed that loss of p53 led to a significant increase of iPS cell reprogramming efficiency by getting rid of senescence barrier that somatic cells encounter during reprogramming process (Hanna et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009). Hanna et al. reported that iPS cell reprogramming is a stochastic process and loss of p53 improves reprogramming efficiency mainly through accelerating cell proliferation. To the contrary to p53 deficiency effects, Nanog expression accelerated iPS cell reprogramming in a predominantly cell-division-rate-independent manner (Hanna et al., 2009). Thus, iPS cell reprogramming could be boosted either by controlling proliferation or affecting the reprogramming process itself. At the same time, there have been several lines of evidences that p53 may be able to directly regulate important stem cell marker gene expression and characteristics of stem cells. First, p53 regulates Oct-4 and Nanog expression (Lin et al., 2005; Qin et al., 2007). Second, p53 activation by nutlin induces rapid differentiation of embryonic stem cells (Maimets et al., 2008). Although the effect of p53 loss on iPS cell reprogramming may be largely through proliferation-dependent manner, it is still possible that p53 directly affect reprogramming process itself at least in part. Therefore, it is necessary to further dissect the precise role of p53 downstream targets in iPS cell reprogramming process. Since c-Myc is a validated target of miR-34 family, a direct target of p53 (Christoffersen et al., 2010; Kong et al., 2008; Lujambio et al., 2008), it is logical to test the role of miR-34 in iPS cell reprogramming process.

1.6 Concluding remarks and project overview

Recent identification of p53/miR-34 regulatory network and its involvement in a number of human cancers opens an opportunity to search for new mechanisms suitable for development of diagnostic, prognostic and therapeutic approaches. High grade serous adenocarcinoma, the most common subtype of EOC, is characterized by frequent *p53* mutations, fast progression and the poorest prognosis. Therefore, it is reasonable to explore the role of miR-34 family in epithelial ovarian carcinogenesis. In Chapter 2, we screened miR-34 expression level in a panel of EOC specimens, and found frequent downregulation of miR-34, which is mostly associated with *p53* mutation but may also occur due to promoter CpG island methylation or copy number variations. Further demonstrating the important role of miR-34 in EOC, we showed that miR-34 family suppresses cell proliferation, migration and invasion of neoplastic cells. We also demonstrated that miR-34-mediated control of cell motility and invasion is likely to occur via regulation of MET, a shared target of miR-34 family. Importantly, our experimental findings were consistent with identification of inverse correlation between MET and miR-34a expression in ovarian cancer patient specimens. In Chapter 3, we further dissected p53/miR-34/MET pathway in primary OSE. This system is of particular value because *p53* mutations are frequent early, likely initiating, events during epithelial ovarian carcinogenesis. Using this model we have shown that p53 plays an important role in regulation of OSE cell motility and invasion. Our finding suggests that early genetic alterations, such as *p53* mutation, may predetermine aggressive cancer traits such as cell migration and invasion. Furthermore, our observation that increased motility and invasion is highly dependent on MET expression supports MET targeting approach as promising cancer therapeutics. In our work we also determined that in addition to miR-34 mediated regulation of MET, p53 regulates MET in miR-34-independent manner, through inhibition of Sp1 DNA binding to MET promoter. Since mutant p53 proteins preserve some binding to SP1, these findings provide mechanistical explanation for the poorest prognosis of patients with p53 null cancer, in which both miR-34 dependent and independent pathways of MET downregulation by p53 are completely inactive. Finally, in Chapter 4, we

describe generation of mice with conditional and conventional knockout of *mir-34a* alone as well as those with entire *mir-34* family. Newly developed *mir-34* conditional knock-out mice allowed us to further confirm miR-34-independent pathway of p53 on MET regulation (described in Chapter 3) and test iPS cell reprogramming efficiency. Characterization of *mir-34* family deficient mice revealed accelerated thymic aplasia and splenic hypoplasia and/or aplasia, suggesting potential roles in hematopoietic system development. Mouse models combined with tissue-specific or inducible Cre expression system will allow us direct genetic testing of miR-34 tumor suppressive functions as well as to establish the biological roles of these highly evolutionary conserved miRNAs during entire mouse ontogenesis.

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CHAPTER 2

FREQUENT DOWNREGULATION OF MIR-34 FAMILY IN HUMAN OVARIAN CANCERS[*]

2.1 Abstract

The miR-34 family is directly transactivated by tumor suppressor p53, which is frequently mutated in human epithelial ovarian cancer (EOC). We hypothesized that miR-34 expression would be decreased in EOC and that reconstituted miR-34 expression might reduce cell proliferation and invasion of EOC cells. miR-34 expression was determined by quantitative reverse transcription-PCR and in situ hybridization in a panel of 83 human EOC samples. Functional characterization of miR-34 was accomplished by reconstitution of miR-34 expression in EOC cells with synthetic pre-miR molecules followed by determining changes in proliferation, apoptosis, and invasion. miR-34a expression is decreased in 100%, and miR-34b*/c in 72%, of EOC with a *p53* mutation, whereas miR-34a is also downregulated in 93% of tumors with wild-type p53. Furthermore, expression of miR-34b*/c is significantly reduced in stage IV tumors compared with stage III (P=0.0171 and P=0.0029, respectively). Additionally, we observed promoter methylation and copy number variations at miR-34. In situ hybridization showed that miR-34a expression is inversely correlated with MET immunohistochemical staining, consistent with translational inhibition by miR-34a. Finally, miR-34 reconstitution experiments in p53 mutant EOC cells resulted in reduced proliferation, motility, and invasion, the latter of which was dependent on MET expression. Our work suggests that miR-34 family plays an important role in

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EOC pathogenesis and reduced expression of miR-34b*/c may be particularly important for progression to the most advanced stages. Part of miR-34 effects on motility and invasion may be explained by regulation of MET, which is frequently overexpressed in EOC.

2.2 Introduction

Ovarian cancer is the most deadly malignancy and will lead to ~14,000 deaths in the United States in 2010 (Jemal et al., 2010). Although survival has increased slightly over the past 25 years, 5-year survival remains below 50%. A major factor for low survival is our poor understanding of the initiating events that lead to ovarian cancer and how the disease progresses. Due to asymptomatic development and few screening options, ~70% of women present at late stages of carcinogenesis. At an advanced stage, treatment options are severely limited, with palliative treatment most often administered in the form of debulking surgery and paclitaxel- and platinum-based therapeutics. However, work over the past decade using human cancer samples and mouse models have revealed new insights into the molecular basis of ovarian cancer, particularly its most common form epithelial ovarian cancer (EOC). For example, it is well established that >50% of highgrade serous-type EOCs contain *p53* mutations and alterations in the RB pathway (reviewed in (Bast et al., 2009) and (Corney et al., 2008)). Consistently, conditional inactivation of p53 and Rb in the mouse ovarian surface epithelium (OSE) leads to development of poorly differentiated serous ovarian adenocarcinomas (Flesken-Nikitin et al., 2003), whereas K-Ras, Pten, and Wnt/β-catenin are implicated in carcinogenesis of the endometrioid EOC subtype (Dinulescu et al., 2005; Wu et al., 2007).

In recent years, the involvement of small noncoding RNAs, called microRNAs (miRNA), in cancers of many types has become unambiguous, including ovarian cancer (Iorio et al., 2007; Zhang et al., 2008). Although the precise roles they play during carcinogenesis are still being dissected, it is clear that miRNAs can act as tumor suppressors and oncogenes by regulating processes such as proliferation and the cell cycle, apoptosis, invasion, and metastasis (reviewed in (Esquela-Kerscher and Slack, 2006)). miRNAs have been found to be dysregulated in cancer by DNA copy number changes and epigenetic alterations, altered processing by the miRNA biogenesis machinery, and through altered transcriptional activation. In particular, the transcription factor and tumor suppressor p53 has been independently shown by several laboratories to directly transactivate genes of the miR-34 family, which is composed of three members (Bommer et al., 2007; Chang et al., 2007; Corney et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007). The gene encoding miR-34a is located on human chromosome 1p36, whereas miR-34b and miR-34c are cotranscribed from one transcription unit on chromosome 11q23. The miR-34 family downregulates numerous important regulatory proteins and thereby presumably mediates tumor suppression (reviewed in (Hermeking, 2007)). Previously, we have shown that conditional inactivation of p53 results in miR-34 downregulation in mouse OSE (Corney et al., 2007). To evaluate the potential roles of miR-34 family in human EOC, we have determined their expression level in a panel of 83 cancer tissues and found that miR-34 expression is frequently decreased in EOC and is associated with metastatic clinical stage and increased expression of receptor protein tyrosine kinase MET. Furthermore, reconstitution of miR-34 expression in EOC cells leads to reduced proliferation and invasion as well as decreased MET levels.

2.3 Material and methods

Clinical samples. Informed consent was obtained from patients undergoing surgery for ovarian cancer at Fox Chase Cancer Center (Philadelphia, PA) and M.D. Anderson Cancer Center (Houston, TX). Sample collection was done after approval by an Institutional Review Board, and a portion of tumor tissue not required for diagnostic purposes was snap frozen in liquid nitrogen and stored at −80°C. Surgical evaluation was used to determine clinical stage and presence of metastases, whereas histopathologic analysis by gynecologic pathologists was done to assess cancer type and subtype. Only tumors found to contain over 70% tumor cells were used in the study, and tissue sample and clinical data were available for 83 patients (Table 2.1). Additional formalin-fixed, paraffin-embedded specimens were obtained from New York-Presbyterian Hospital/Weill Cornell Medical Center (New York, NY).

miR-34 nomenclature. miRNA nomenclature has recently been revised such that the miR-34b sequence has been renamed miR-34b* (i.e., the passenger strand; (Landgraf et al., 2007)). Nevertheless, “star” or passenger strands of the miRNA duplex have previously been shown to be biologically important (e.g., miR-199a* and miR-10*; (Migliore et al., 2008; Stark et al., 2007)). Although both miR-34b strands are likely to be functional, the miR-34b strand is not predicted to bind the 3′-untranslated region of MET and our studies have therefore focused on the miR-34b* strand. It is worth noting that our quantitative reverse transcription-PCR (qRT-PCR) data show that both strands are present at equal quantities and are highly correlated (Figure 2.2); they therefore might be renamed miR-34b-3p and miR-34b-5p, respectively, consistent with mouse miR-34b nomenclature.

p53 mutation screening. The IARC protocol was followed for *p53* mutation screening. DNA was isolated by DNeasy Mini kit (Qiagen), and exons 4 to 11, including splice junctions, were amplified by PCR and sequenced with both forward and reverse primers (Table 2.2). In the case sequencing data were unclear, T-vector cloning was done, and three clones were sequenced by T7 and SP6 primers.

Quantitative reverse transcription-PCR. Total RNA was isolated using mirVana miRNA Isolation kit (Ambion) according to the manufacturer's protocol, and RNA concentration and purity were determined by NanoDrop analysis. Stem-loop qRT-PCR for mature miR-34 and miR-199a* miRNAs was done as previously described (Chen et al., 2005). For MET qRT-PCR, cDNA was prepared from 100 ng total RNA using SuperScript III (Invitrogen) and amplified with Taqman primer/probes. All PCRs were done in triplicate on an ABI 7500 Real-Time PCR System (Applied Biosystems, Inc.), and miRNA and mRNA expression was normalized to RNU6B and GAPDH, respectively, using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

MET immunohistochemistry. Paraffin sections of formalinfixed tissue were stained according to modified avidin-biotinperoxidase technique (Nikitin and Lee, 1996). The antibody used for detection of MET was CVD13 from Zymed Laboratories (dilution, 1:200).

In situ hybridization. Detection of miR-34 in a panel of serous adenocarcinomas was done by the protocol adapted from Nelson et al. (Nelson et al., 2006). To prevent the loss of miRNAs, we additionally applied 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) fixation as described by Pena et al. (Pena et al., 2009). In brief, 4-µm-thick sections of formalin fixed, paraffin-

embedded material were deparaffinized, rehydrated, and fixed with EDC. After 1-h prehybridization, a digoxigenin (DIG)–labeled locked nucleic acid (LNA) probe (Exiqon) was hybridized to proteinase K–treated sections at 56°C for 16 h. Slides were then incubated with anti-DIG-AP antibody (Roche), and miRNA expression was detected by nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate method. Methyl green was used for nucleic counterstaining.

Quantitative PCR. For copy number variation analysis, DNA was isolated with a DNeasy DNA mini kit (Qiagen, Valencia, CA) and 60 ng DNA amplified with custom TaqMan real time primers and probes that were designed to amplify across the *mir-34a* locus. Copy number was calculated by normalizing to *LINE1* retrotransposon gene copy number, which is maintained at relatively constant levels in neoplastic and normal tissues (Kuo et al., 2009; Wang et al., 2002), using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Primer sequences are given in Table 2.2. A total of 30 serous, 1 mucinous and 2 tumors of undetermined histology with clinical characteristics similar to the entire cohort were analyzed. Samples Pooled human placental DNA served as a wild type control (Bioline, Taunton, MA).

Methylation-specific PCR analysis. Genomic DNA isolated as described above was treated with bisulfite using the EZ DNA methylation kit (Zymo Research, Orange, CA). A total of 27 serous, 1 mucinous and 2 undetermined EOC tumors with clinical characteristics similar to the entire cohort were analyzed. The modified DNA was eluted with a final volume of 16 µl, and 1 µl to 2 µl were used for the methylation specific polymerase chain reactions (MSP). In case of *mir-34a*, amplification conditions were 5 min at 95°C; 2 cycles of 20 sec at 95°C, 30 sec at 68°C, and 30 sec at 72°C; followed by 2 cycles with 66°C annealing temperature, then 34 cycles with 65°C annealing temperature and a 4 min final extension at 72°C. For *mir-34b*/c*, amplification

conditions were 37 cycles of 20 sec at 95°C, 30 sec at 61°C and 30 sec at 72°C, 4 minutes final extension. Primers were used were previously reported (Lodygin et al., 2008; Lujambio et al., 2008) and are listed in Table 2.2. PCR products were separated by electrophoresis on an 8% polyacrylamide gel and band intensities visually scored.

Cell culture experiments. Human *p53* mutant ovarian adenocarcinoma cell line SKOV-3 was obtained from the American Type Culture Collection and maintained according to the supplier's directions. For proliferation and apoptosis analysis, cells were seeded in triplicate in 24-well plates and the next day transfected with Pre-miR synthetic miRNA molecules (Ambion, Austin, TX) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Proliferation was assessed by BrdU incorporation and apoptosis assessed by cleaved caspase-3 staining 48 hours posttransfection as previously described (Nikitin and Lee, 1996; Zhou et al., 2006). For the apoptosis assay, cells were treated with 5 μM camptothecin for 17 hours to induce apoptosis. Invasion assays were performed in a chamber containing an 8 μm pore-size PET membrane coated with a uniform layer of BD Matrigel™ Basement Membrane (BD Bioscience, San Jose, CA). For MET knock down experiments, 2 μmol/mL of MET siRNA (sc-29397, Santa Cruz, Santa Cruz, CA) was transfected. 24 hours after transfection, 5 x 10^3 SKOV-3 cells per each well were seeded in a control insert or Matrigel insert in serum free media and translocated toward complete growth media containing 20 ng/μl hepatocyte growth factor (HGF). 20 hours after additional culture, invading cells were stained by Multiple Staining Solution (Polysciences, Inc., Warrington, PA) and counted under the microscope. All experiments were performed in triplicate and three different fields of each well were counted.

Western blotting. Cells were lysed with RIPA buffer (25mM Tris, pH 8.2, 50mM NaCl, 0.1% SDS, 0.5% Nonidet P-40, 0.5% deoxycholate) containing protease inhibitor cocktail (Roche,

Indianapolis, IN). Lysates were resolved by 8% SDS-PAGE, transferred to PVDF membrane, and probed with antibodies against MET (clone C-28, Santa Cruz, Santa Cruz, CA), CDK4 (clone C-22, Santa Cruz, Santa Cruz, CA) and GAPDH (clone 6C5, Advanced Immunochemical, Long Beach, CA).

Statistical analysis. Statistical tests used were two-sided Student's t tests, with Welch's correction for unequal variance, where appropriate, using InStat 3.05 and Prism 4.03 software (GraphPad, Inc.).

2.4 Results

2.4.1 miR-34 expression is reduced in EOC and is correlated with metastatic stage.

To determine miR-34 family expression in EOC, we isolated total RNA from 83 EOC samples and compared expression levels to that in six wild-type OSE primary cell samples (Table 2.1). We observed significantly reduced expression for all three family members in EOC compared with wild-type (Figure 2.1A), with miR-34a most significantly reduced by 21.2-fold ($P < 0.0001$), whereas miR-34b* and miR-34c were reduced by 2.3-fold ($P = 0.0172$) and 3.4-fold ($P = 0.0002$), respectively. It has been recently reported that Drosha and Dicer expression is deregulated in EOC (8, 25). To test whether alteration in miR-34 expression can be explained by Drosha/Dicer-mediated global changes in miRNA processing, we determined miR-199a* expression, which is deregulated in EOC (Iorio et al., 2007; Yang et al., 2008). miR-199a* expression seems to be elevated, although this is not statistically significant (Figure 2.3). No

Table 2.1 Characteristics of 83 patients with EOC

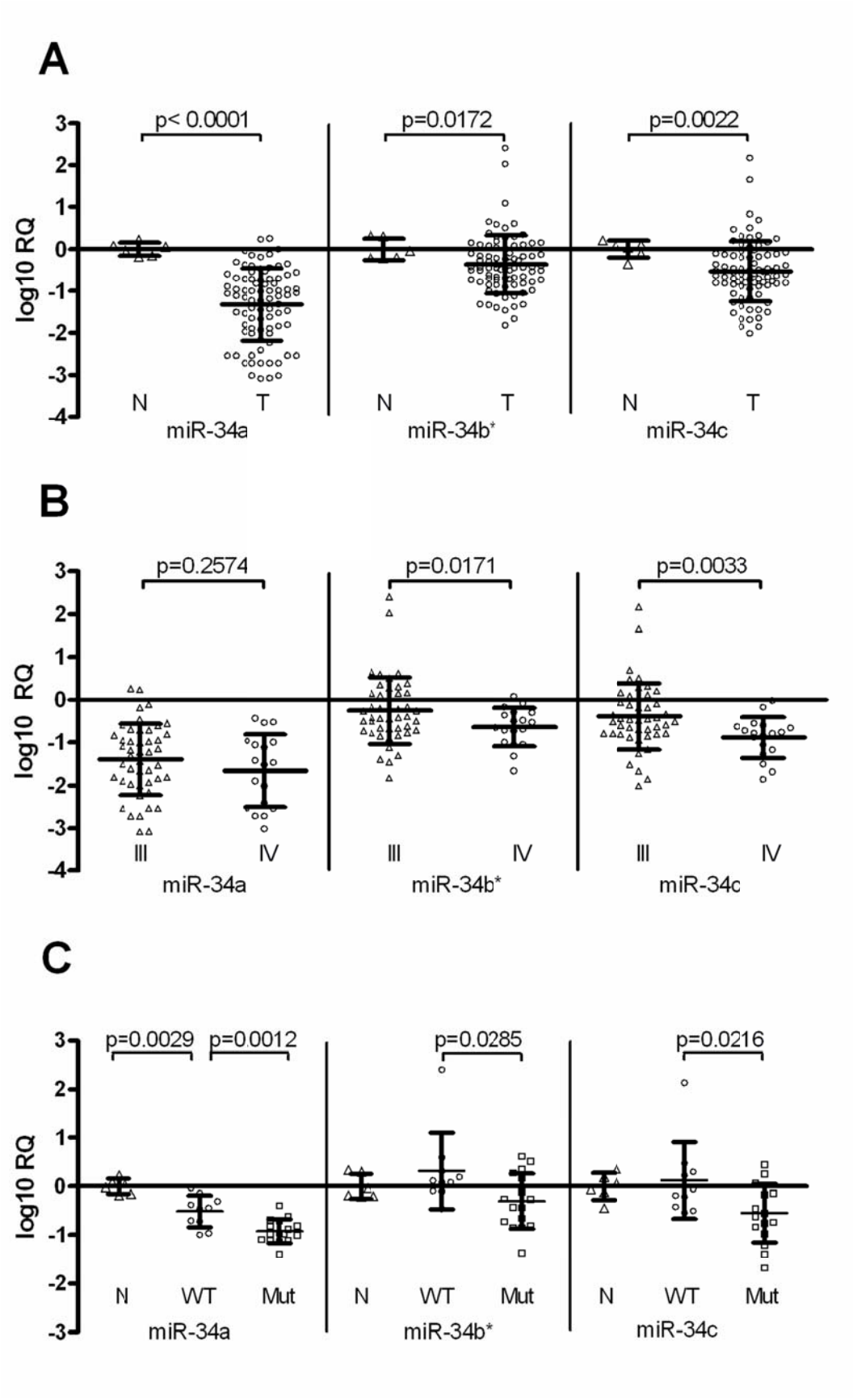
Characteristics	Value
Age (y)	
Mean	60.8
Range	28-87
Race (%)	
White	64 (77.1)
African American	5 (6)
Other non-white	6 (7.2)
Unknown	2 (2.4)
Tumor stage (%)	
I	2 (2.4)
II	4 (4.8)
III	46 (55.4)
IV	18 (21.7)
TX	13 (15.7)
Histology (%)	
Serous	62 (74.7)
Mucinous	2 (2.4)
Endometrioid	3 (3.6)
Clear cell	1 (1.2)
Adenocarcinoma, NOS/undifferentiated	4 (4.8)
Mixed	11 (13.3)

Abbreviation : NOS, not otherwise specified

Table 2.2 Sequences for primers used in this study

Gene		Forward primer	Reverse primer	Probe
miR-34a		AGTGTCTTAGCTGGTTGTTGTGA	GCAGCACTTCTAGGGCAGTAT	TTGCTGATTGCTTCCTTACTATTGC
LINE1		GCTCCTGAATGACTACTGGGTACA	GTGTCTTTGTTCTCGTTGGTTTCAAAATAAA	ACGAAATGAAGGCAGA
p53	Exon 4	TGCTCTTTTCACCCATCTAC	ATACGGCCAGGCATTGAAGT	N/A
	Exon 5	TTCAACTCTGTCTCCTTCCT	CAGCCCTGTCGTCTCTCCAG	N/A
	Exon 6	GCCTCTGATTCCTCACTGAT	TTAACCCCTCCTCCCAGAGA	N/A
	Exon 7	AGGCGCACTGGCCTCATCTT	TGTGCAGGGTGGCAAGTGGC	N/A
	Exon 8/9	TTGGGAGTAGATGGAGCCT	AGTGTTAGACTGGAAACTTT	N/A
	Exon 10	CAATTGTAACTTGAACCATC	GGATGAGAATGGAATCCTAT	N/A
	Exon 11	AGACCCTCTCACTCATGTGA	TGACGCACACCTATTGCAAG	N/A
miR-34a methylated		GGTTTTGGGTAGGCGCGTTTC	TCCTCATCCCCTTCACCGCCG	N/A
miR-34a unmethylated		IIGGTTTTGGGTAGGTGTGTTTT	AATCCTCATCCCCTTCACCACCA	N/A
miR-34b/c* methylated		TTTAGTTACGCGTGTTGTGC	ACTACAACTCCCGAACGATC	N/A
miR-34b/c* unmethylated		TGGTTTAGTTATGTGTGTTGTGT	CAACTACAACTCCCAAACAATCC	N/A

Figure 2.1 miR-34 is downregulated in EOC and associated with metastatic clinical stage. A. qRT-PCR analysis reveals significantly reduced miR-34a, miR-34b* and miR-34c expression in a panel of EOC samples (n=83; O) relative to normal OSE samples (n=6; ∆) (P<0.0001, P=0.0172 and P=0.0022, respectively). B. Ovarian cancer stage was determined by surgical evaluation during cryoreduction/debulking surgery. miR-34a, miR-34b* and miR-34c expression is shown for stages 1 and 2 combined (n=6; ∆), stage 3 (n= 46; O) and stage 4 (n= 18; □). miR-34b* and miR-34c expression is significantly decreased in stage 4 (distant metastasis) compared to stage 3 (localized to peritoneum; P=0.0171 and 0.0029, respectively). C. Adenocarcinomas (serous type, n=26) and wild type OSE samples (n=6) were analyzed for *p53* mutational status by direct sequencing and correlated with miR-34 expression. While both *p53* wild type (WT; O) and *p53* mutant (Mut; □) EOC samples show significantly reduced miR-34a expression compared to wild type OSE (N; ∆), EOC samples with mutant *p53* demonstrate most downregulated expression for miR-34a, miR-34b* and miR-34c (P=0.0012, 0.0285 and 0.0216, respectively). Bars, SD. Kindly provided by Dr. David Corney.


A
p< 0.0001
p=0.0172
p=0.0022
log10 RQ
3
2
1
0
-1
-2
-3
-4
N
T
N
T
N
T
miR-34a
miR-34b*
miR-34c
B
p=0.2574
p=0.0171
p=0.0033
log10 RQ
III
IV
III
IV
III
IV
miR-34a
miR-34b*
miR-34c
C
p=0.0029
p=0.0012
p=0.0285
p=0.0216
log10 RQ
N
WT
Mut
N
WT
Mut
N
WT
Mut
miR-34a
miR-34b*
miR-34c

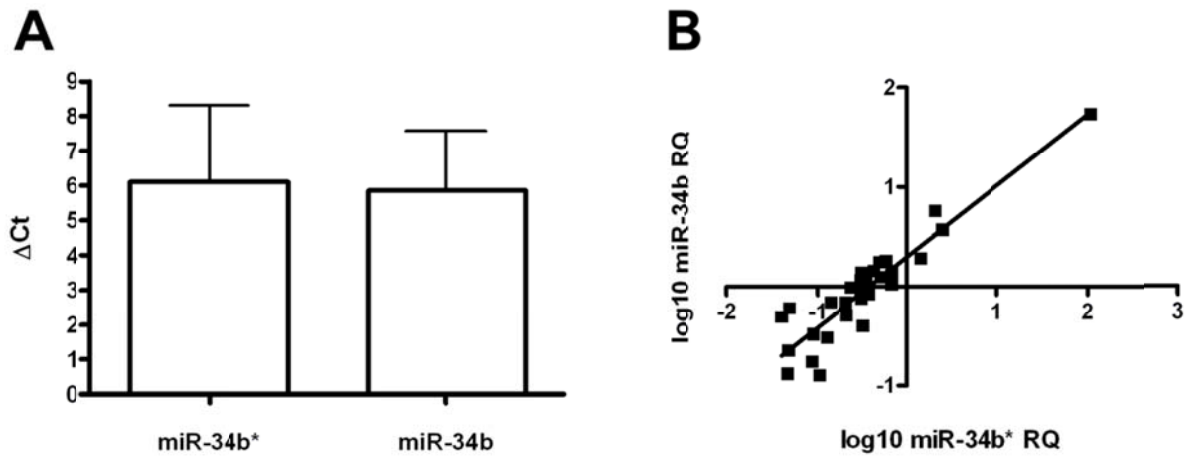


Figure 2.2 miR-34b and miR-34b* are present in equal amounts and are highly correlated. A. miR-34b* and miR-34b strands are present in equal quantities in EOC (n= 30, P=0.1764, 2-tailed paired t-test. B. miR-34b* and miR-34b show significant correlation in their expression (r^2= 0.8497, P<0.0001). Bars, SD. Kindly provided by Dr. David Corney.

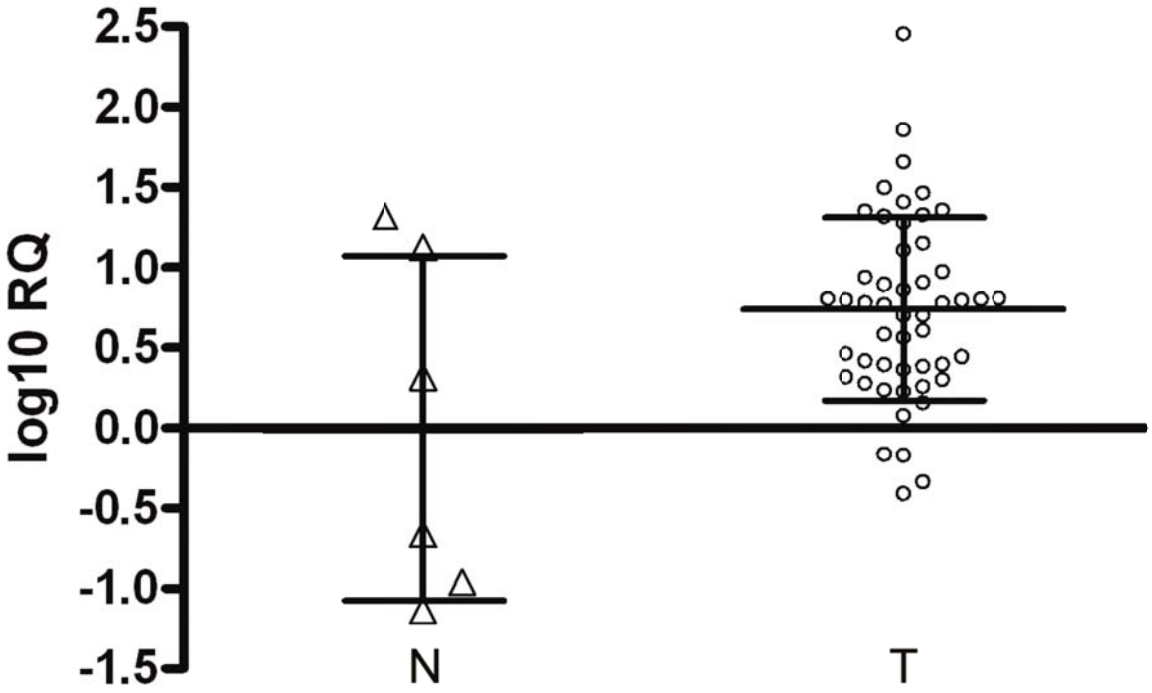


Figure 2.3 miR-199a* is not significantly altered in EOC. qRT-PCR profiling demonstrates no significant alteration in miR-199a* expression between EOC samples (n= 50, O) and normal OSE (n= 6, ∆) (p=0.1578). Bars, SD. Kindly provided by Dr. David Corney.

significant differences in expression of miR-34 family members were detected among different histologic types of EOC, with an exception of significantly reduced miR-34a expression in endometrioid type as compared with serous adenocarcinoma (Figure 2.4).

Tumor staging is linked to survival, with stage III tumors having tumor cell dissemination in the peritoneum, whereas stage IV tumors have distant metastasis, commonly to liver, and is indicative of poor prognosis. We compared gene expression in stage III and IV tumors and observed significantly reduced miR-34b* and miR-34c expression in stage IV tumors (P = 0.0171 and P = 0.0033, respectively; Figure 2.1B), suggesting that miR-34b* and miR-34c may be involved in metastatic progression. Interestingly, however, change in miR-34a expression was not statistically significant (P = 0.2574).

2.4.2 Decreased miR-34 expression is associated with p53 mutation.

Mutation of *p53* is a common event in many human cancers but is particularly common in high-grade serous EOC (Corney et al., 2008). We therefore took a subset of our serous EOC samples (n = 26) and sequenced p53 exons 4 to 11, where >99% of *p53* mutations are located (Table 2.3). We have found that although miR-34a expression is reduced in both samples with wild-type or mutant p53, patients with mutant p53 show significantly lower expression of all miR-34 family members than patients with wild-type p53 (P = 0.0012, 0.0285, and 0.0216 for miR-34a, miR-34b*, and miR-34c, respectively; Figure 2.1C).

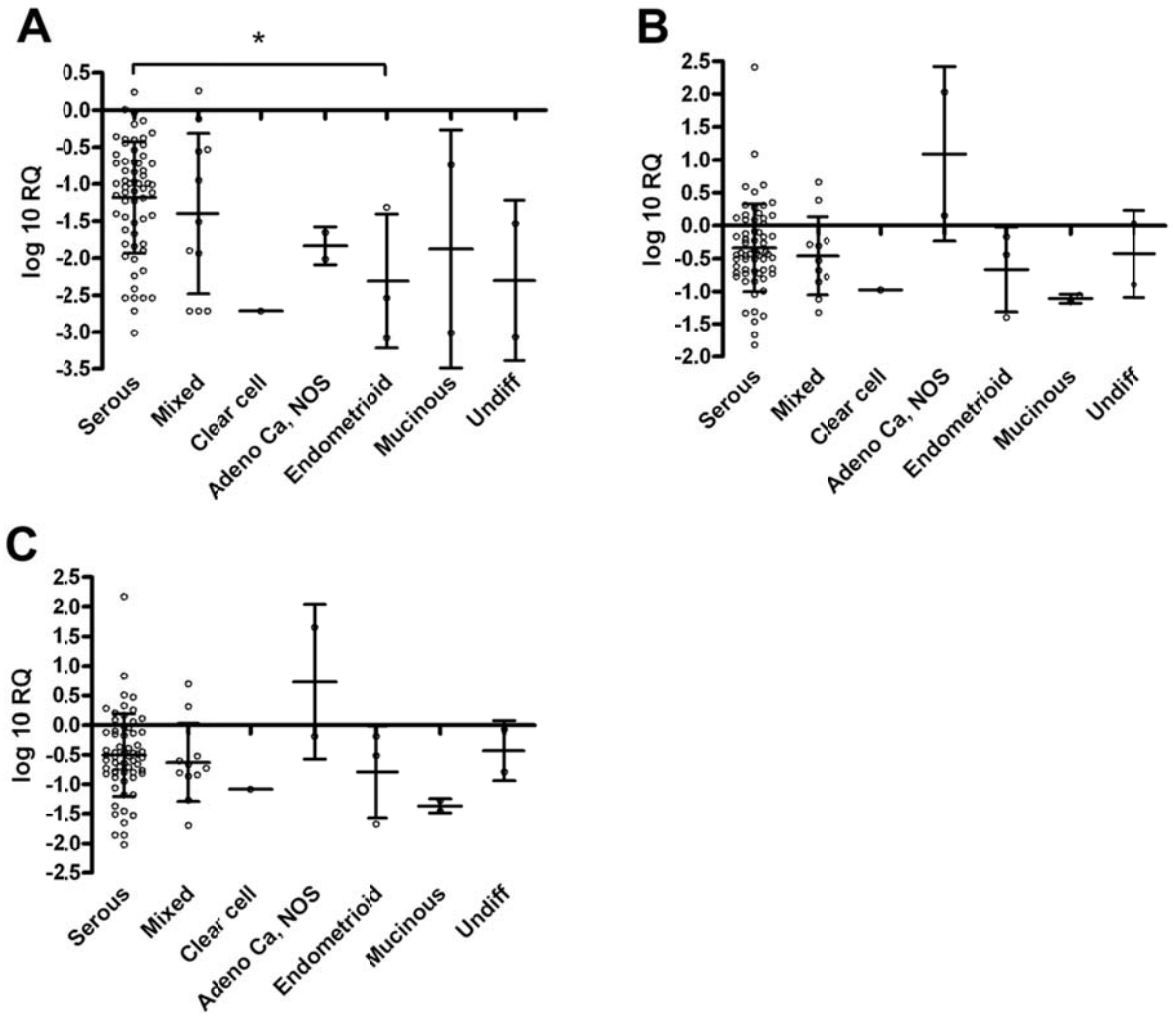


Figure 2.4 miR-34 family expression in different EOC subtypes. qRT-PCR analysis reveals significantly reduced miR-34a expression in endometrioid compared to serous EOC (P=0.0144) (A), while no significant alteration in miR-34b* or miR-34c expression is observed (B, C). Adeno Ca, adenocarcinoma. Bars, SD. Kindly provided by David Corney.

Table 2.3 *p53* mutation status of human samples

Case no.	Mutation type	exon	codon	Change	Wild-type	Mutated	Wild-type AA	Mutated AA
OvCa 1	Wild-type							
OvCa 2	Wild-type							
OvCa 6	Wild-type							
OvCa 11	Wild-type							
OvCa 12	Wild-type							
OvCa 21	Wild-type							
OvCa 24	Wild-type							
OvCa 27	Wild-type							
OvCa 28	Wild-type							
OvCa 31	Wild-type							
OvCa 4	Missense	7	237	G>A	ATG	ATA	Met	Ile
OvCa 7	Missense	6/7	220/229	A>G / T>C	TAT/TGT	TGT/CGT	Tyr/Cys	Cys/Arg
OvCa 8	Missense	5	176	G>T	TGC	TTC	Cys	Phe
OvCa 10	Missense	11	375	A>G	CAG	CGG	Gln	Arg
OvCa 14	Missense	7	244	G>A	GGC	GAC	Gly	Asp
OvCa 15	Missense	7	237	G>T	ATG	ATT	Met	Ile
OvCa 17	Missense	8	286	G>A	GAA	AAA	Glu	Lys
OvCa 18	Missense	5	151	C>T	CCC	TCC	Pro	Ser
OvCa 25	Missense	7	248	G>A	CGG	CAG	Arg	Glu
OvCa 30	Missense	11	385	T>C	TTC	TCC	Phe	Ser
OvCa 32	Missense	7	241	C>G	TCC	TGC	Ser	Cys
OvCa 13	Nonsense	5	144	C>T	CAG	TAG	Gln	STOP
OvCa 33	Nonsense	4	107	C>A	TAC	TAA	Tyr	STOP
OvCa 16	Insertion	5	169	+ T	ATG			
OvCa 22	Insertion	4	99	+ C	TCC			
OvCa 34	Deletion	7	241	- T	TCC			

2.4.3 Regulation of miR-34 by promoter methylation and copy number alterations.

Promoters of both *mir-34a* and *mir-34b*/c* are located in CpG islands, and methylation has been reported to regulate miR-34a expression in several cancer cell lines and primary prostate tumors and melanomas, whereas miR-34b*/c expression in colorectal cancer is also epigenetically regulated (Lodygin et al., 2008; Toyota et al., 2008). Such methylation has not been reported in ovarian cancer, so we set out to determine the frequency of such methylation by methylation-specific PCR analysis. Methylation at the *mir-34a* and *mir-34b*/c* loci was observed in 27% (8 of 30) and 47% (14 of 30) of EOC samples, respectively (Figure 2.5A). All samples (8 of 8) with *mir-34a* methylation show reduced miR-34a expression, whereas 57% (8 of 14) of samples with methylation at *mir-34b*/c* show reduced miR-34b*/c expression.

Classification of samples based on *p53* mutation status revealed that 21% (3 of 14) and 50% (7 of 14) of mutant p53 samples show promoter methylation at *mir-34a* and *mir-34b*/c*, respectively, whereas for samples with wild-type p53, 38% (5 of 13) and 46% (6 of 13) show methylation, respectively.

We next raised the question of whether loss of heterozygosity or copy number alterations could be responsible for reduced miR-34a expression and designed custom Taqman primer and probes to amplify the *mir-34a* locus in a qPCR assay. Reduced copy number at the *mir-34a* locus was observed in 39% (13 of 33) of EOC samples (Figure 2.5B), of which 92% (12 of 13) had reduced miR-34a expression. Three of these 12 samples (25%) with reduced *mir-34a* copy number and expression showed no *p53* mutation or promoter methylation. Taken together, reduced miR-34a expression is associated with *p53* mutation, *mir-34a* promoter methylation, and/or copy number variation in 82% (27 of 33) of EOC samples.

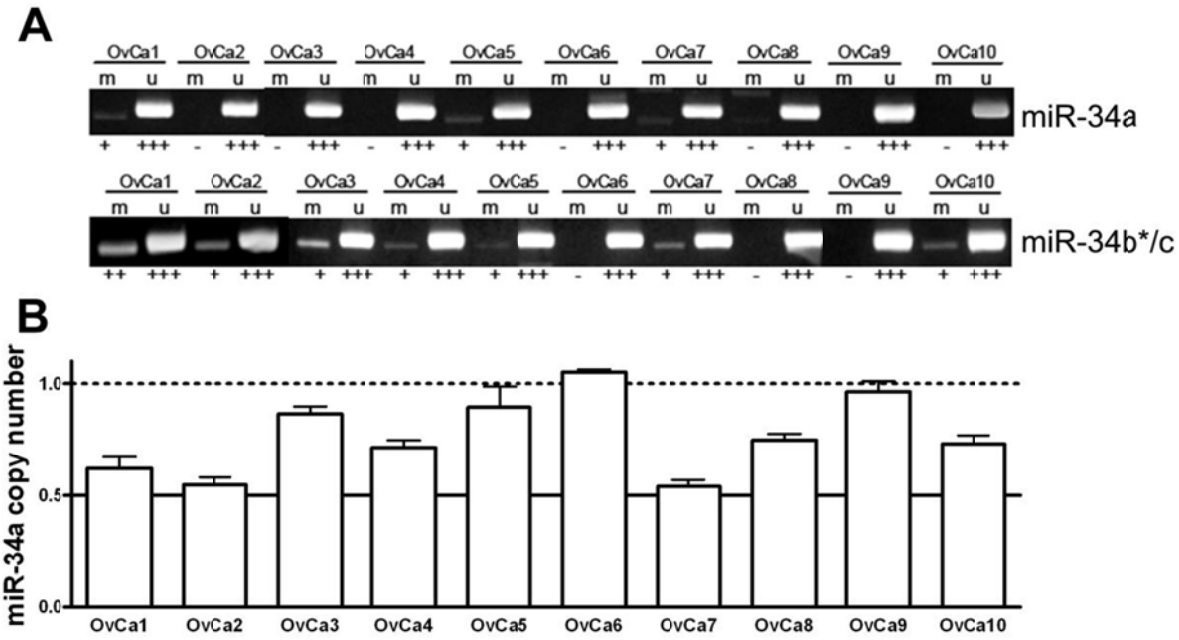


Figure 2.5 *miR-34* promoter methylation and copy number variations are common in EOC. A and B, representative examples of *miR-34a* and *miR-34b*/c* promoter methylation determined by methylation-specific PCR with primers specific for methylated (M) and unmethylated (U) DNA (A) and copy number changes at the *miR-34a* locus determined by qPCR (B). Bars, SD. Kindly provided by Dr. Heiko Hermeking and Markus Vogt.

2.4.4 miR-34 and MET expression in EOC paraffin sections.

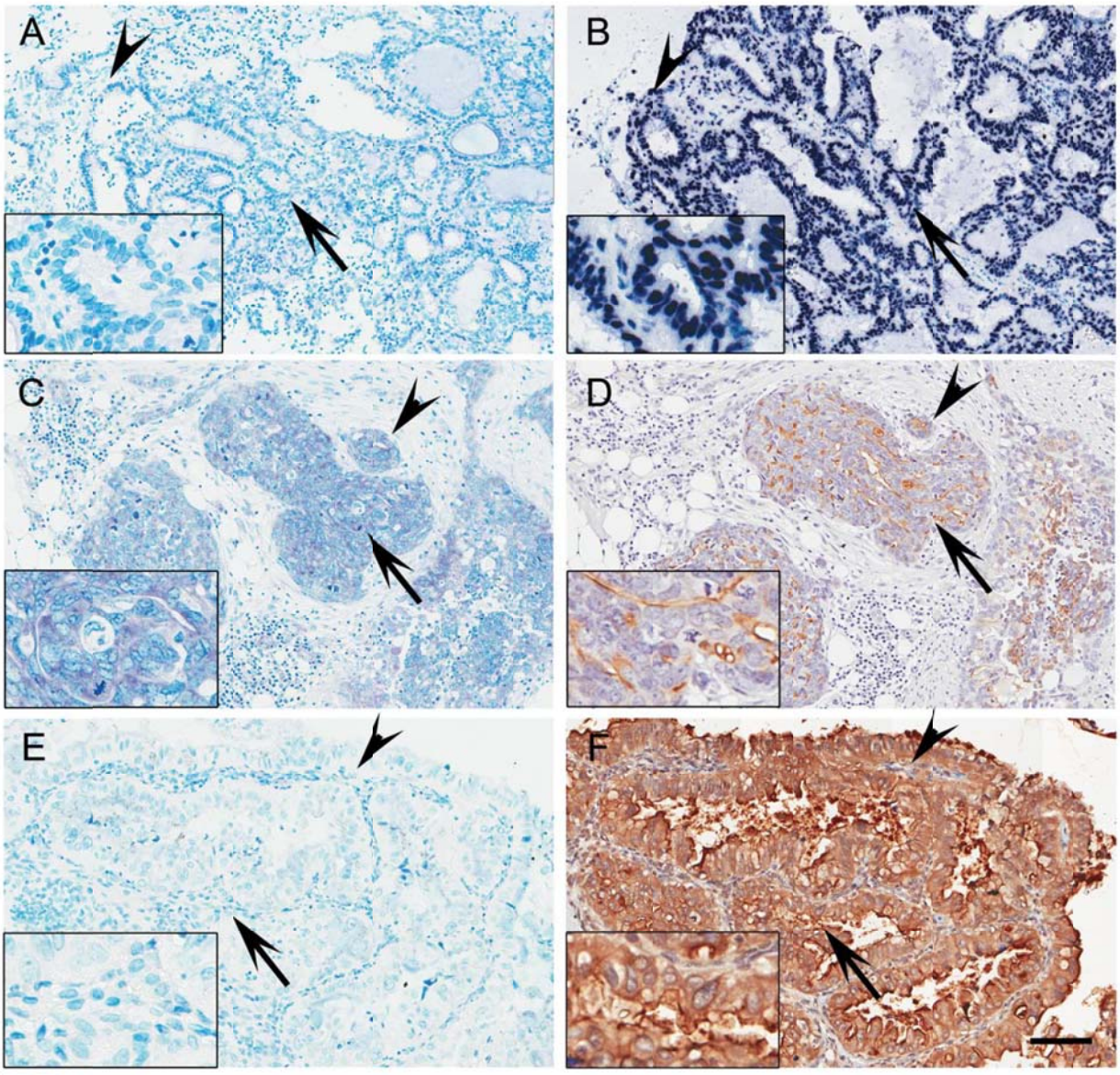
To explore miR-34 expression in EOC tissue, we did miRNA in situ hybridization with paraffin-embedded tissue sections of human ovarian cancer. In the case of miR-34a probe, we observed positive signal in cytoplasm compared with control probe, whereas U6 small nuclear RNA was exclusively expressed in the nucleus as expected (Figure 2.6A-C). We tested a total of 21 cases of serous EOC with LNA miR-34a probe. Consistent with qRT-PCR data, 85.7% (18 of 21) of cases have weak or undetectable miR-34a expression.

One of the shared targets of miR-34 family is the receptor tyrosine kinase MET according to bioinformatic assessment and luciferase assays (He et al., 2007; Migliore et al., 2008). Furthermore, the majority of EOCs express elevated levels of MET (Auersperg et al., 2001). Thus, we decided to compare MET expression level in parallel sections of 17 cases with semiquantitative immunohistochemical analysis. EOC cases expressing moderate to strong miR-34a (Figure 2.6C) had a relatively weak expression of MET (Figure 2.6D). On the contrary, low miR-34a–expressing EOC (Figure 2.6E) had strong expression of MET (Figure 2.6F). Based on semiquantitative analysis, expression of miR-34a and MET had statistically significant inverse correlation ($r = -0.5898$; $P = 0.0162$), confirming that miR-34a might play a role in regulating MET expression in EOC.

2.4.5 miR-34 reduces migration, invasion, and proliferation in EOC cells.

To determine the role of miR-34 in human ovarian cancer, we transfected synthetic miR-34 molecules either separately or in combination into SKOV-3, p53-null human ovarian adenocarc-

Figure 2.6 miR-34a expression is inversely associated with MET expression in EOC. Sections of formalin-fixed, paraffin-embedded human EOC specimens were hybridized with DIG-labeled LNA control probe (A), U6 small nuclear RNA probe (B), and miR-34a probe (C and E) or immunostained with MET antibody (D and F). C and D, EOC with strong miR-34a expression shows relatively low level of MET expression. E and F, on the contrary, EOC with weak miR-34a expression has strong staining for MET. Insets show high magnification of areas indicated with arrows. Similar structures of parallel sections (A and B, C and D, and E and F) are indicated with arrowheads. Methyl green and hematoxylin were used for counterstaining of in situ hybridization and immunostaining, respectively. Scale bar, 100 μm. Parts D and F kindly provided by Andres Matoso.

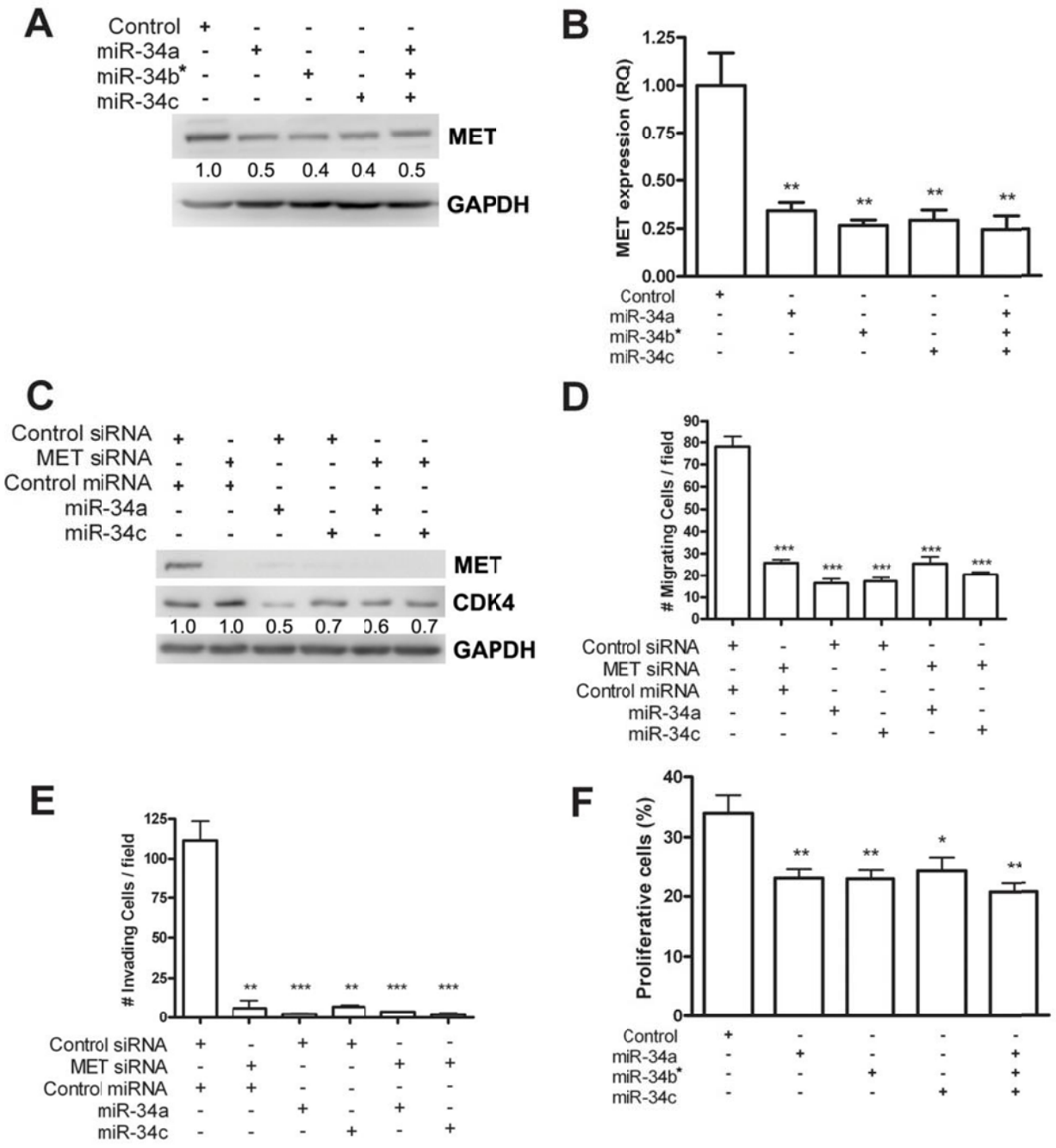

A
B
C
D
E
F

inoma cells (Figure 2.7 and 2.8). SKOV-3 cells express low endogenous levels of all three miR-34 family members (Corney et al., 2007; Zhang et al., 2008) and are therefore well suited to test functions of miR-34. We transfected SKOV-3 cells with 15 nmol/L miR-34 individually or 5 nmol/L combined and observed reduced amounts of MET protein and mRNA (Figure 2.7A and B). Even more significant reduction of MET levels was observed after transfection with 30 nmol/L miR-34 (Figure 2.7C).

The miR-34 family has been shown to reduce cell invasion in gastric and hepatocellular carcinoma cells, at least partially through downregulation of MET (Li et al., 2009; Migliore et al., 2008). To examine the role of miR-34 family in invasion and motility in ovarian cancer, we did Transwell motility and Matrigel invasion assays with miR-34 family and/or MET small interfering RNA (siRNA)–transfected SKOV-3 cells. Notably, whereas MET knockdown was observed after MET siRNA treatment, cyclin-dependent kinase 4 (CDK4), which is another target of miR-34 family, was not affected by MET siRNA but only after miR-34 family transfection (Figure 2.7C). As expected, individual miR-34 reconstitution by transfection caused significant reduction in motility and invasion in the presence of the MET ligand, hepatocyte growth factor (Figure 2.7D and E; Figure 2.8). However, when miR-34 and MET siRNA were transfected together, no further reduction was observed, showing that MET downregulation by miR-34 is largely responsible for the reduced invasion.

Next, we asked whether miR-34 family reconstitution reduces cell proliferation because miR-34 family also can target cell cycle–related genes such as CDK4 (Figure 2.7C). Transfection of SKOV-3 cells with either 15 nmol/L miR-34a, miR-34b*, or miR-34c reduced proliferation by ~30% compared with control-transfected cells ($P = 0.0044$, 0.0042, and 0.0106, respectively; Figure 2.7F). We next treated cells with a combination of 5 nmol/L of each miR-34

Figure 2.7 miR-34 reconstitution decreases migration, invasion and proliferation in EOC cells. A and B. Individual miR-34 family member (15 nM) or entire miR-34 family (5 nM each) were transfected and statistically significant reduction in mRNA observed for each treatment (miR-34a, p=0.028; miR-34b*, p=0.0017; miR-34c, P=0.0022; entire miR-34 family, P=0.0020), although reconstitution of entire miR-34 family does not further downregulate MET expression. C. MET siRNA and/or indicated miR-34 precursor molecule (30 nM) were transfected in SKOV-3 cell line. Transfected cell lysates were probed against MET, CDK4 and GAPDH in Western blot analysis. MET siRNA induced strong knockdown of MET protein with no effect on CDK4, while miR-34 downregulated CDK4 as well as MET. D and E, MET siRNA and/or miR-34 transfection induced significant reduction of cell migration (MET siRNA, P=0.0005; miR-34a, P=0.0003; miR-34c, P=0.0003; MET siRNA and miR-34a, P=0.0008; MET siRNA and miR-34c, P=0.0003) and invasion (MET siRNA, P=0.0013; miR-34a, P=0.0009; miR-34c, P=0.0011; MET siRNA and miR-34a, P=0.0009; MET siRNA and miR-34c, P=0.0009). F. Quantitative assessment of proliferation by BrdU incorporation 48 hours after transfection of SKOV-3 cells with either 15 nM synthetic miR-34a, miR-34b* or miR-34c Pre-miR individually, or 5 nM of each Pre-miR concurrently (miR-34a, P=0.0044; miR-34b*, P=0.0042; miR-34c, P=0.0106; miR-34 in combination, P=0.0021). Bars, SD. Part F kindly provided by David Corney.


A
Control + - - - -
miR-34a - + - - +
miR-34b* - - + - +
miR-34c - - - + +
MET
1.0 0.5 0.4 0.4 0.5
GAPDH
B
MET expression (RQ)
1.25
1.00
0.75
0.50
0.25
0.00
**
Control + - - - -
miR-34a - + - - +
miR-34b* - - + - +
miR-34c - - - + +
C
Control siRNA + - + + - -
MET siRNA - + - - + +
Control miRNA + + - - - -
miR-34a - - + - + -
miR-34c - - - + - +
MET
CDK4
1.0 1.0 0.5 0.7 0.6 0.7
GAPDH
D
Migrating Cells / field
90
80
70
60
50
40
30
20
10
0
***
Control siRNA + - + + - -
MET siRNA - + - - + +
Control miRNA + + - - - -
miR-34a - - + - + -
miR-34c - - - + - +
E
Invading Cells / field
125
100
75
50
25
0
**
***
Control siRNA + - + + - -
MET siRNA - + - - + +
Control miRNA + + - - - -
miR-34a - - + - + -
miR-34c - - - + - +
F
Proliferative cells (%)
40
30
20
10
0
**
*
Control + - - - -
miR-34a - + - - +
miR-34b* - - + - +
miR-34c - - - + +

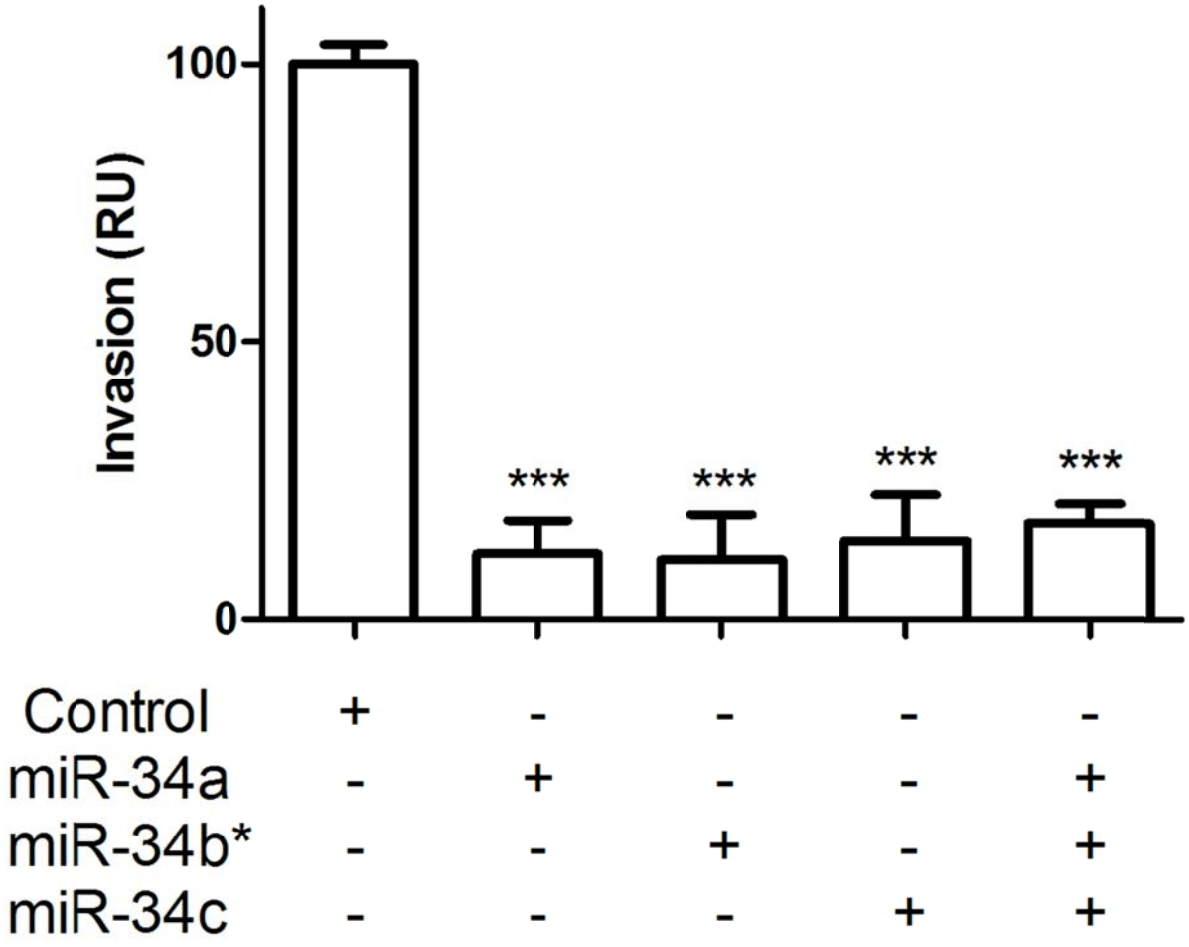


Figure 2.8 miR-34 family reduces invasion. Individual miR-34 family member (30 nM) or entire miR-34 family (10 nM each) were transfected in SKOV-3 cell line. miR-34 family transfection induced significant reduction of invasion (miR-34a, $P<0.0001$; miR-34b*, $P<0.0001$; miR-34c, $P<0.0001$; all three family members, $p<0.0001$). Bars, SD.

family member to determine whether additional suppression could be achieved due to sequence, and presumably target, differences of miR-34 family members. Although the percentage of proliferative cells was reduced to 20.7% (P = 0.0021, compared with control), the difference in reduction compared with miR-34c transfection individually was not statistically significant (P = 0.0876). Additionally, we assessed the amount of apoptosis in miR-34–transfected cells by determination of cleaved caspase-3 staining (Figure 2.9). No significant change in number of apoptotic cells was observed in p53 mutant SKOV-3, consistent with miR-34–induced apoptosis being p53 dependent (Yamakuchi et al., 2008).

2.5 Discussion

Previously, we showed that miR-34b-5p and miR-34c expression is reduced in a p53-dependent manner in a mouse model of EOC, whereas others also reported reduced miR-34 expression in a variety of cell lines and mouse models (Bommer et al., 2007; Chang et al., 2007; Corney et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007). These results led us to question the involvement of miR-34 in human EOC, and we show here that miR-34 family expression is also significantly reduced in human EOC, particularly in patients with *p53* mutations.

Recently, the involvement of Drosha and Dicer in EOC has been reported, linking reduced expression of these proteins to poor outcome (Merritt et al., 2008). Yet, although reduced Drosha/Dicer processing might be expected to lead to globally decreased miRNA expression, this does not seem to account for all miRNA expression defects, given that miR-199a* expression is modestly increased (Figure 2.3). Moreover, unlike miR-34a, miR-34b*/c expression is not reduced in tumors with wild-type p53. This discrepancy between miR-34a and

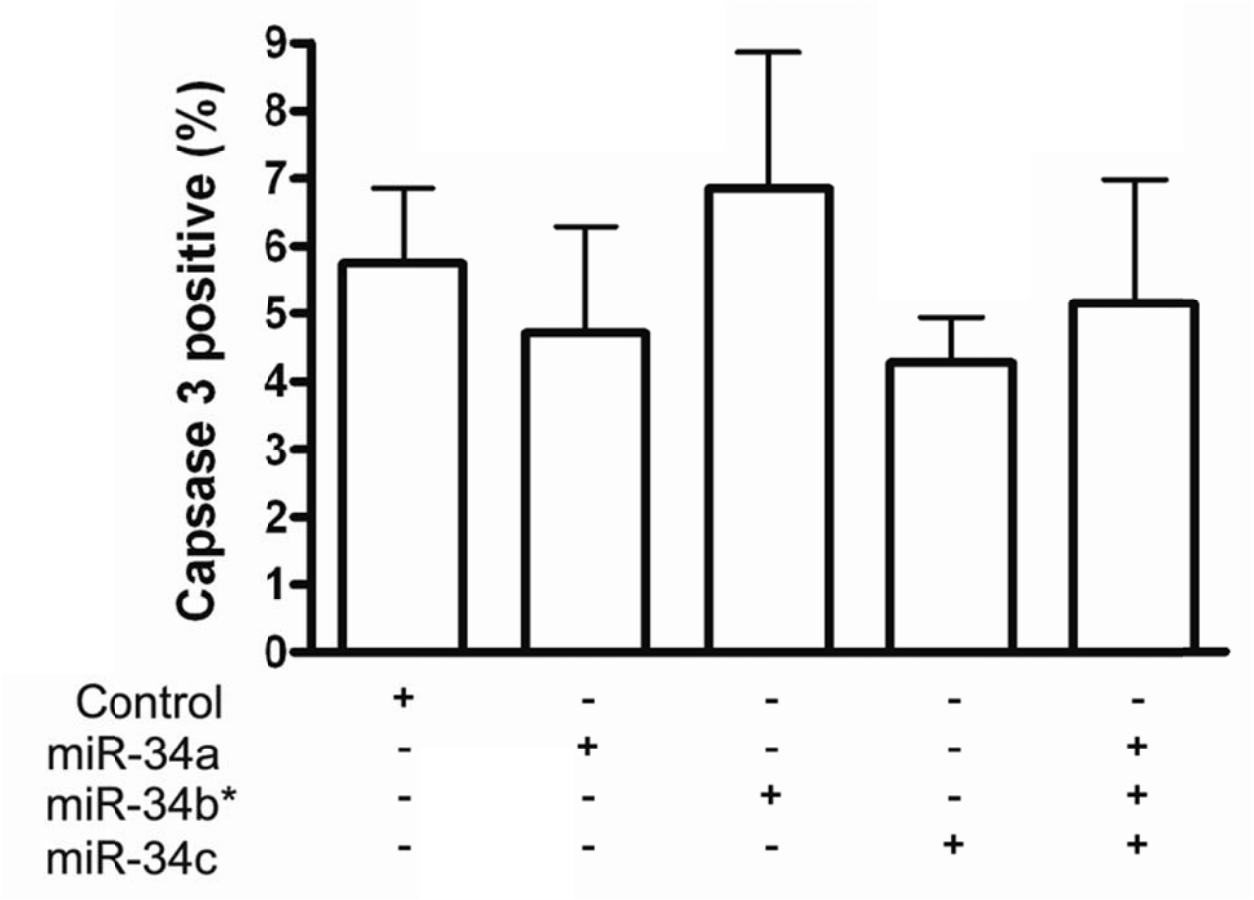


Figure 2.9 Apoptosis is unaffected by miR-34 reconstitution. SKOV-3 cells were transfected with either 15 nM miR-34a, miR-34b* or miR-34c Pre-miR individually, or 5 nM of each concurrently. Number of apoptotic cells was determined by cleaved caspase 3 immunostaining 48 hours after transfection and 17 hours after treatment with 5 μM camptothecin to induce apoptosis. Compared to cells transfected with 15 nM non-targeting control Pre-miR, no significant alteration in number of cells undergoing apoptosis was observed (miR-34a, P=0.4066; miR-34b*, P=0.4513; miR-34c, P=0.1209; all three family members, P=0.6554). Bars, SD. Kindly provided by David Corney.

miR-34b*/c expression suggests that, in addition to shared p53-dependent transactivation, control mechanisms unique to miR-34a are altered in tumors with wild-type p53. Underlining differences between the two *mir-34* loci is our observation that expression of miR-34b*/c, but not expression of miR-34a, is significantly associated with stage IV distant metastatic disease (Figure 2.1B).

To understand the cause of these differences, we investigated the role of promoter methylation and copy number variations of *mir-34*. *mir-34* promoter methylation has been reported in several tumor types (Lodygin et al., 2008; Toyota et al., 2008), whereas megabase pair deletions at chromosome 1p36 containing the *mir-34a* locus have been identified in seven low-grade serous carcinomas (Kuo et al., 2009) and in neuroblastoma (Welch et al., 2007). Indeed, promoter methylation was observed at *mir-34a* and *mir-34b*/c* in 27% and 47% of EOC samples, respectively. Furthermore, reduced *mir-34a* copy number was observed in 39% of samples. However, there was no direct correlation between methylation or copy number and miR-34 expression levels. There are several possible explanations that may account for these data. Firstly, although our *p53* sequencing data identified *p53* mutations with high confidence, mutation in genes that regulate p53 may be involved, such as MDM2, which posttranslationally silences p53 through ubiquitinylation (Haupt et al., 1997; Kubbutat et al., 1997), whereas p53 may itself be epigenetically silenced (Kang et al., 2001). Secondly, many miRNAs, including miR-34a, have been shown by Chang et al. (Chang et al., 2008) to be suppressed by c-Myc, an oncogene frequently overexpressed in multiple tumor types, including EOC. Additionally, it is likely that additional transcription factors regulate miR-34 expression. Recently, Christoffersen et al. (Christoffersen et al., 2010) studied human primary hTERT-immortalized TIG3 fibroblasts and observed p53-independent transcription of miR-34a. Oncogene-induced senescence mediated through B-RAF activation induces miR-34a expression in cells treated with a p53 siRNA or a p53 dominant-negative variant. Through chromatin immunoprecipitation experiments,

the authors show that the ETS oncogene family member and transcription factor ELK1 binds a conserved region in the *mir-34a* promoter.

Clearly, future studies are required to obtain a more complete understanding of the regulation of miR-34 expression in both normal development and disease. However, the observation of B-RAF–induced miR-34a expression through oncogene-induced senescence raises an important question about the etiology of EOC. It has been hypothesized that low- and high-grade serous tumors have distinct precursor lesions (Corney et al., 2008; Singer et al., 2005), with *p53* mutations rarely found in low-grade tumors but common in high-grade tumors. Interestingly, and further emphasizing the differing molecular defects between the two tumor types, activating B-RAF mutations are found exclusively in low-grade serous EOC (Shih and Kurman, 2004; Singer et al., 2003). Together, this suggests that activation of miR-34a transcription by B-RAF/ELK1 and p53 in low-grade serous EOC induces senescence and prevents progression to high-grade disease. In contrast, a lack of B-RAF mutations combined with frequent *p53* mutation in high-grade serous EOC would seem to largely eliminate miR-34 expression, and subsequently, tumor suppression capability is lost. Additionally, whereas *p53* mutation has no effect on miR-34b*/c methylation, miR-34a methylation is more common in samples with wild-type p53, consistent with a requirement for diminished p53–miR-34 activity to progress to carcinoma. Together, these observations suggest that high-grade tumors arise from a population of cells with mutated p53 but wild-type B-RAF. Our data showing correlation of miR-34b*/c expression with metastatic disease suggest that these two miRNAs will be useful as a prognostic marker. This observation is in a good agreement with a recent report that low miR-34a levels are correlated with increased probability of relapse in non–small cell lung carcinoma (Gallardo et al., 2009) and reinforces the importance of decreased miR-34 expression. Future studies based on complete follow-up data will determine if miR-34 expression correlates with survival of EOC patients.

Our functional studies of miR-34 reconstitution suggest therapeutic applications too. miRNAs represent attractive candidates for gene therapy approaches for several reasons. Computationally, individual miRNAs have been predicted to target tens or hundreds of mRNAs for translational repression. Indeed, one miRNA may regulate many targets. For example, in the case of miR-223 in neutrophils, hundreds of proteins are directly repressed, which has a significant effect on phenotype, despite protein repression being relatively modest (Baek et al., 2008; Johnnidis et al., 2008). It is noteworthy that microarray experiments done after miR-34 reconstitution in cancer cell lines revealed highly significant alterations that are clustered to important biological pathways (e.g., cell cycle pathway genes; (Bommer et al., 2007; He et al., 2007)). These data suggest that reconstituted expression of a downregulated miR-34 might reset or otherwise induce normal function of these gene networks.

Due to its large number of transcriptional targets, p53 has also been an attractive candidate for gene therapy. It was therefore disappointing that p53 gene therapy in EOC failed in phase II/III clinical trials (Zeimet and Marth, 2003). Yet, whereas p53 therapy may have failed due to p53 degradation through MDM2 and dominant-negative tetramer formation, gene therapy with a p53-independent miR-34 transgene under control of a strong promoter would not be expected to face either of these problems. Furthermore, miR-34 may be more attractive than p53 due to its small size, making it more amenable to packaging in viral and nonviral technologies. No less of importance, several EOCs do not harbor *p53* mutations but express low levels of miR-34, which could be corrected. Thus, it would be interesting to study the consequence of miR-34 delivery to EOC cells with wild-type p53. However, currently characterized EOC cell lines carrying wild-type p53, such as the serous adenocarcinoma cell line A2780 and clear cell carcinoma cell line TOV-21G (He et al., 2007; Migliore et al., 2008), show high levels of expression of endogenous miR-34.

MET is one of few common targets for all three miR-34 family members. Taking into account that the majority of EOCs express elevated levels of MET (Auersperg et al., 2001), we have tested the role of all miR-34 family members in repression of MET in EOC cells by using Western blotting, invasion, and motility assays, finding that all three members inhibit MET. Small-molecule MET inhibitors are now under clinical trials for several cancers (Comoglio et al., 2008), and Sawada et al. (Sawada et al., 2007) showed that MET siRNA could reduce adhesion, invasion, metastasis, and tumor burden in intraperitoneal ovarian cancer xenograft model but did not affect proliferation.

On the contrary, miR-34 therapeutics may have advantages compared with siRNA approach because miR-34 is capable of regulating cell proliferation as well as invasion through targeting several target genes in addition to MET, such as MYC and E2F3. This is underscored by our experiment showing reduced CDK4 protein after miR-34 treatment, but not MET siRNA.

Taken together, our data show the frequent reduction of miR-34 family expression in EOC and its functional properties as an inhibitor of proliferation and invasion. This miRNA family is therefore an attractive candidate gene for further studies aimed at better understanding of disease pathogenesis and development of novel therapeutic approaches.

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CHAPTER 3

WILD-TYPE P53 CONTROLS CELL MOTILITY AND INVASION BY DUAL REGULATION OF MET EXPRESSION [*]

3.1 Abstract

Recent observations suggest that p53 mutations are responsible not only for growth of primary tumors but also for their dissemination. However, mechanisms involved in p53-mediated control of cell motility and invasion remain poorly understood. By using the primary ovarian surface epithelium cell culture, we show that conditional inactivation of *p53* or expression of its mutant forms results in overexpression of MET receptor tyrosine kinase, a crucial regulator of invasive growth. At the same time, cells acquire increased MET-dependent motility and invasion. Wild-type p53 negatively regulates MET expression by two mechanisms: (1) transactivation of MET-targeting miR-34 and (2) inhibition of SP1 binding to *MET* promoter. Both mechanisms are not functional in p53 absence, while mutant p53 proteins retain partial *MET* promoter suppression. Accordingly, MET overexpression, cell motility and invasion are particularly high in *p53* null cells. These results identify MET as a critical effector of p53 and suggest that inhibition of MET may be effective anti-metastatic approach to treat cancers with *p53* mutations. They also show that the extent of advanced cancer traits, such as invasion, may be determined by alterations in individual components of p53/MET regulatory network.

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3.2 Introduction

Transcriptional factor p53 provides integrated responses to implement cell cycle arrest, senescence, differentiation, inhibition of cancer metabolism or induction of the apoptotic cascade (Vousden and Prives, 2009). Mutations of *p53* occur in about 50% of all cancers and result in loss of its function, either by null phenotype or dominant-negative effect. Additionally, some mutations result in new activities of p53, known as gain-of-function mutations (Brosh and Rotter, 2009). Recent observations indicate that *p53* mutations affect cell motility and invasion, key features of metastasis (Adorno et al., 2009; Alexandrova et al., 2000; Gadea et al., 2007; Muller et al., 2009; Muller et al., 2011; Wang et al., 2009). Better understanding of mechanisms of p53-dependent effects on cell motility and invasion should lead to development of approaches aimed towards correction of aberrant p53 signaling not only for suppressing growth of primary tumors but also for preventing their dissemination.

A signaling conduit known to play a critical role in invasion and metastasis is the MET pathway (Mazzone and Comoglio, 2006). The *MET* proto-oncogene encodes a transmembrane receptor protein tyrosine kinase, whose overexpression is associated with poor prognosis in a broad variety of cancers (Comoglio et al., 2008; Sawada et al., 2007). Inhibition of MET functions has been shown to be effective in animal models and is among the most promising candidates for targeted therapy (Comoglio et al., 2008).

Previously it has been reported that MET is overexpressed in tumors of p53-deficient mice and in Li-Fraumeni patients (Rong et al., 1995). This observation is consistent with recent reports that MET represents one of the common targets for miR-34 family (Corney et al., 2010; He et al., 2007; Migliore et al., 2008). Genes encoding for miR-34 family have been identified as direct targets of p53 transactivation (reviewed in (Hermeking, 2007). At the same time, it has

been reported that mouse *Met* promoter has a putative p53 responsive element and that *Met* promoter activity is activated by p53 through DNA binding to p53 consensus sequence (Seol et al., 1999). Thus, the role of MET in p53-dependent invasion remains uncertain.

Because many cancers are genomically unstable and separation of critical alterations from "genetic noise" may be a daunting task in cells derived from advanced stages of the disease, we have used a model of conditional *p53* inactivation in the primary ovarian surface epithelium (OSE), transformation of which leads to epithelial ovarian cancer (EOC) (Corney et al., 2007; Flesken-Nikitin et al., 2003). This system is highly clinically relevant, because *p53* mutations are by far the most frequent alterations in high-grade serous adenocarcinoma of the ovary (Ahmed et al., 2010), are detected in the stage 1 of those cancers and in adjacent dysplastic lesions (Berchuck et al., 1994; Leitao et al., 2004), and their presence correlates with metastatic potential (Bast et al., 2009). MET overexpression is also associated with poor prognosis of EOC patients and targeting the MET pathway has been reported to suppress EOC in mouse models (Sawada et al., 2007).

We report that MET is a critical player in p53-mediated control of motility and invasion, and show that such control includes miR-34-independent regulation of MET expression by p53, in addition to earlier described MET targeting by miR-34. Alterations in individual components of p53/MET regulatory network may affect the extent of cancer invasion.

3.3 Methods

Experimental animals. Mice with floxed copies of *p53*, *Rb1*, *Met* and *p53* mutant allele genes and genotyping PCR information were described elsewhere (Huh et al., 2004; Jonkers et al.,

2001; Marino et al., 2000; Olive et al., 2004). Z/EG reporter mice (Novak et al., 2000) were purchased from the Jackson Laboratory. All mice were maintained identically following recommendations of the Cornell Institutional Laboratory Animal Use and Care Committee.

Cell culture. Primary mouse OSE cells and OSE cell lines (OSN1; p53 and Rb null, OSN2; p53 null) were isolated and cultured as previously described (Corney et al., 2007; Flesken-Nikitin et al., 2003; Flesken-Nikitin et al., 2007). Human ovarian cancer cell lines SKOV-3 (p53 null), OVCAR-3 (p53 mutant), human lung cancer cell line NCI-H1299 (p53 null) and human colon cancer cell line HCT116 (p53 wild-type) were obtained from the American Type Culture Collection (Manassas, VA) and maintained according to the supplier's directions. Human ovarian cancer cell lines OVCA432 (p53 mutant) and OVCA433 (p53 wild-type) were maintained as described previously (Mok et al., 1995; Tsao et al., 1995). Transfection was performed by using lipofectamine 2000 (Invitrogen, Carlsbad, CA), and control (sc-37007) and p53 siRNA (sc-29436 and sc-29435 for mouse and human respectively) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mithramycin A and doxorubicin were purchased from Sigma, and HGF from R&D Biosystems (Minneapolis, MN). In experiments with conditional gene inactivation the efficiency of adenoviral infection was over 90% according to use of adenoviral Cre:EGFP fusion protein expression system. Cre-mediated gene excision was at similar levels, according to PCR genotyping (Figure 3.6B-C) and our previous experiments with Rosa26STOPflLacZ reporter (Flesken-Nikitin et al., 2003).

Cell proliferation and senescence assays. Proliferation of OSE was assessed by BrdU incorporation as described elsewhere (Flesken-Nikitin et al., 2003). For detection of senescence-associated β-galactosidase OSE cells were fixed with 3% formaldehyde, rinsed

several times with PBS and incubated in staining-solution (4.2 mM citric acid, 12.5 mM sodium-phosphate, 158 mM sodium chloride, 0.21 mM magnesium chloride, 2.21 mg/ml potassium ferrocyanide, 1.68 mg/ml potassium ferricyanid, 1 mg/ml X-Gal, pH 6.0) for 24 hrs at 37 °C.

Conditional gene inactivation and mRNA microarray profiling studies. Primary OSE cells carrying conditional gene alleles were passaged 3 times and treated with recombinant adenoviruses essentially as described previously (Corney et al., 2007; Flesken-Nikitin et al., 2003). Cells were collected 2 passages after infection and processed for mRNA isolation and assessment of mRNA profiles. Total RNA was prepared using RNeasy lipid tissue mini kit (Qiagen, Valencia, CA). The generation of biotin-labeled cRNA fragmentation, hybridization to the Affymetrix murine U74Av2 arrays (Santa Clara, CA), washing, and scanning were done according to Affymetrix protocols. The gene expression signals from Affymetrix GCOS software were normalized by scaling each GeneChip to a target signal of 500. Log ratio was calculated with the average value of control groups. Analysis was performed by using Significant Analysis of Microarrays (SAM) software (http://www-stat.stanford.edu/~tibs/SAM/) with 2 fold setting and 1% False Discovery Rate (FDR) of genes (Tusher et al., 2001). Identified profile was visualized in TreeView software (http://rana.lbl.gov).

Trans-infundibular administration of adenovirus, collection of histological materials and immunohistochemistry. All procedures were performed as described previously (Flesken-Nikitin et al., 2003). Double immunofluorescence staining of frozen sections was done with rabbit polyclonal antibody to EGFP (Clontech, Mountain View, CA, 632377, 1:100) or to rabbit polyclonal MET (Santa Cruz Biotechnology, Santa Cruz, CA, SP260, 1:100), followed by fluorescein (FITC)-conjugated anti-rabbit and rhodamine (TRITC)–conjugated anti-rabbit

secondary antibodies (Jackson ImmunoResearch Laboratories, 1:50). To stain cell nuclei, sections were incubated with a 10 μg/mL solution of 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO) for 4 min.

Wound healing, time-lapse microscopy, migration and invasion assay. For wound healing assay, OSE cells were infected with adenovirus and cultured in 10 cm gelatin-coated dish to confluence. Cells were scraped with p200 tip, and fresh medium supplemented. Digital images of the wound were taken at 0, 12 and 24 hours after scraping. Percentage of closed wound area was measured by using TScratch software (Geback et al., 2009). For the time-lapse microscopy, cells were grown until confluent and then partially scratched by a 2 mm wide plastic space. Twenty four hrs after scratching, complete culture medium were replaced with CO_2-independent medium supplemented with 5% fetal bovine serum. Cell movements were captured using a camera on a microscope at 20x objective in a 37°C chamber. Image of the cells was taken every 15 minutes for 5 hrs for a total of 21 images. Individual cell movements were tracked by using Image J, Multitrack software. Migration and invasion assays were performed as described in (Corney et al., 2010).

Quantitative real-time RT-PCR. Total RNA was isolated using Qiazol (Qiagen, Valencia, CA) according to manufacturer's protocol. Total RNA was reverse transcribed using SuperScript III (Invitrogen, Carlsbad, CA) and oligo-dT primers. The PCR reactions were done in a 30-μl volume in a 96-well plate using pre-developed FAM TaqMan probes (Applied Biosystems, Foster City, CA). Mouse and human Gapdh were used as endogenous reference control (pre-developed TaqMan assay from Applied Biosystems). Cycling parameters were as follows: 95˚C

for 10 min. followed by 40 cycles of 95˚C for 15 seconds and 60˚C for 1 min. Increase in real-time fluorescence was measured by an ABI7500 qPCR system and relative fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Promoter analysis. pGL2-3.1MET construct consists of 3 Kb upstream sequence and 131 bp 5'UTR of human *MET* gene. pGL2-0.65MET construct consists of 653 bp upstream sequence including 131 bp 5'-UTR (Gambarotta et al., 1996). 0.37MET construct was cloned from -512 to -136 of MET 3.1 fragment. pGL2-0.27MET construct consists of 136 bp of MET upstream sequence and 131 bp of 5'-UTR. The corresponding human sequence of previously reported p53 binding site (Seol et al., 1999) in mouse *Met* promoter (from -289 to -256) was mutated. 5'GGACGGACAGCACGCGAGGCAGACAGACACGTG3' sequence was changed into 5'GGACGGACAGCACGCGAGGCAGACAGACAAAAG3'. To measure luciferase activity, cells were processed with the Luciferase Reporter Assay System (Promega, Madison WI) according to manufacturer's instructions, and 10 µl of cell lysates was used to determine reporter enzyme activity using Lumat LB 9501 luminometer (Berthold, Bad Wilbad, Germany). Lipofectamin 2000 reagent (Invitrogen, Carlsbad, CA) was used for the transfection following manufacturer's recommendations. pORF-hp53 was purchased from InvivoGen (San Diego, CA). Pre-miR miRNA precursor molecules of miR-34a, b and c, as well as control pre-miR were purchased from Ambion (Austin, TX). Each experiment was performed in triplicates. Luciferase activities were normalized to total protein concentrations. BCA method (Pierce, Rockford, IL) was applied for measuring protein concentrations.

Western blot analysis. Cell lysates were prepared using RIPA buffer (Tris-HCl 50 mM, pH 7.4; NP-40 1%; Na-deoxicholate 0.25%; NaCl 150 mM; EDTA 1 mM; PMSF 1 mM; Aprotinin, leupeptin, pepstatin: 1 µg/ml each; Na3VO4 1 mM; NaF 1 mM), separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Millipore, Billerica, MA). The membrane was incubated overnight at 4˚C with antibodies to detect MET (SP 260 for mouse and C28 for human, Santa Cruz Biotechnology, 1:1000 dilution for both), p53 (FL 393 from Santa Cruz Bitechnology, 1:1000 dilution), CD44 (2C5, R&D Systems, Inc., Minneapolis, MN), Hif-1α (H1alpha67 from NOVUS biologicals, Littleton, CO), phospho-MET (Y1234/1235 from Cell Signaling Technology, Danvers, MA), SP1 (Millipore, Billercia, MA) and Gapdh (Advanced Immunohistochemical Inc.; Long Beach, CA; 1:3000 dilution) followed by incubation for one hr at room temperature with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and developed using chemiluminescence substrate (SuperSignal West Pico from Pierce; Rockford, IL).

Co-immunoprecipitation. Lysates of were prepared in modified Lysis250 buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5mM EDTA, 0.1% NP-40 100 mM). The antibodies used for immunoprecipitation were polyclonal anti-SP1 (Millipore, Billercia, MA), polyclonal anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal anti-p53 (Cell Signaling Technology, Danvers, MA). Cell extracts were incubated at 4°C overnight with 2 µg of corresponding antibodies, followed by incubation with 20 µl of Sepharose G bead (GammaBind Plus Sepharose, GE Healthcare, Niskayuna, NY) for 2 hrs at 4°C. Immunoprecipitates were isolated by centrifugation and followed by 5 times washing with lysis buffer. Samples were resuspended in the 2X sample buffer, subjected to 12% SDS-polyacrylamide gel

electrophoresis, transferred onto a PVDF membrane and the immunoprecipitated proteins were detected by Western blotting.

Chromatin immunoprecipitation (ChIP). SKOV-3 cells were grown on 10 cm dishes and transfected with control and p53 expression vector respectively. One day after transfection, 1% formaldehyde was added for 10 min at 37°C to cross-link proteins to DNA. Cells were washed two times with ice-cold 1X PBS, scraped and lysed with SDS lysis buffer (Millipore, Billercia, MA) in the presence of protease inhibitors cocktail. The lysates were sonicated to shear DNA to lengths between 200 bp and 1000 bp. After 10 fold dilution of the sonicated cell supernatants in ChIP dilution buffer (Millipore, Billercia, MA) supplemented with the protease inhibitors cocktail (Roche, Indianapolis, IN), immunoprecipitations were carried out overnight at 4°C with rotation by using 4 µg of polyclonal anti-SP1 (Millipore, Billercia, MA) antibody. Protein A Agarose/Salmon sperm DNA were added to the chromatin-antibody complexes for 1h at 4°C with rotation. Washing and reverse crosslinking procedures were done according to the manufacturer's guide (Millipore, Billercia, MA). PCR purification kit (Qiagen, Valencia, CA) was used for DNA elution from protein-DNA complexes. Immunoprecipitated DNA was analyzed for *MET* gene promoter sequence by PCR with proximal (5'-CAG GGC GAG AAA ACT TCT CCA CCT A-3' and 5'-GCA CCG CCA GGC GAC CAG ACT GA-3') and distal (5'-TCA GTC TTG GAG ATG TTG TTC CCA A-3' and 5'-CCA GCA TGC AGA AGT TGG TGC TGA-3') primer sets. Corresponding proximal region of mouse *Met* promoter sequence was analyzed by PCR with proximal primers (5'-GCT TTG CGC GGG TGA CTT TG-3' and 5'-AGC ACG TGT CTG TTC GTC CCT G-3') as previously described (Morozov et al., 2007). Advantage GC genomic LA polymerase mix (Clontech, Mountain View, CA) was used for PCR amplification of *MET* promoter region due to high GC contents. PCR products were separated on a 2% agarose gel and stained with ethidium bromide and were observed under UV light. The proximal region of

survivin promoter was used as positive control for SP1 binding (Esteve et al., 2007). Three independent ChIP assays were performed. The band intensity of PCR results were measured by ImageJ software, and normalized by 2% input control.

Statistics. Statistical analyses were performed with InStat 3.10 and Prism 5.01 software (GraphPad, Inc., San Diego, CA).

3.4 Results

3.4.1 p53 inactivation leads to MET overexpression.

To evaluate immediate transcriptome changes associated with *p53* inactivation, we conducted mRNA microarray analysis of primary OSE cells after acute inactivation of *p53, Rb* or both *p53* and *Rb* concomitantly (Figure 3.1A and Figure 3.2). Interestingly, in addition to the expected targets of p53 and Rb/E2f signaling, upregulation of *Met* proto-oncogene was detected as a consequence of *p53* and *p53/Rb* inactivation, but not of inactivation of *Rb* alone (Figure 3.2). According to qRT-PCR (Figure 3.1B) and Western blot analysis (Figure 3.1C), MET expression levels continued to rise for 72 hours after gene inactivation and were particularly high in neoplastic OSE cell lines (over 40 passages) deficient for p53 (OSN2) or p53 and Rb (OSN1). Consistently, p53 knockdown in human ovarian cancer cells OVCA433 and colon cancer cells

Figure 3.1 Inactivation of *p53* leads to increase of MET expression. (A) Outline of experiments. After Ad-*Cre*-mediated *p53* inactivation, the mRNA expression profile was generated followed by qRT-PCR and Western blot validation of identified targets and elucidation of their functions by experimental testing. (B-C) Levels of MET mRNA and protein in primary OSE cells before and after *p53* inactivation or *p53* and *Rb* inactivation according to qRT-PCR and Western blot analysis, respectively. Neoplastic OSE cell lines (over 40 passages) deficient for p53 (OSN2), or p53 and Rb (OSN1), have particularly high levels of MET. qRT-PCR data: n=4, mean ± S.D. (D) Representative examples (upper and lower rows) of MET expression in the OSE of $p53^{fl/fl}Rb^{fl/f}$ Z/EG mice 72 hrs after exposure to either Ad-*Cre* (upper) or Ad-Blank (lower) delivered by trans-oviductal intrabursal injection. Z/EG reporter indicates Cre-*loxP* mediated recombination by expression of EGFP. Note co-localization (arrows, yellow) of MET (red) and EGFP (green) in OSE cells that underwent Cre-mediated recombination. Arrows indicate OSE. Double immunofluorescence. DAPI counterstaining. Scale bar, 100 μm. Kindly provided by Dr. Andres Matoso.

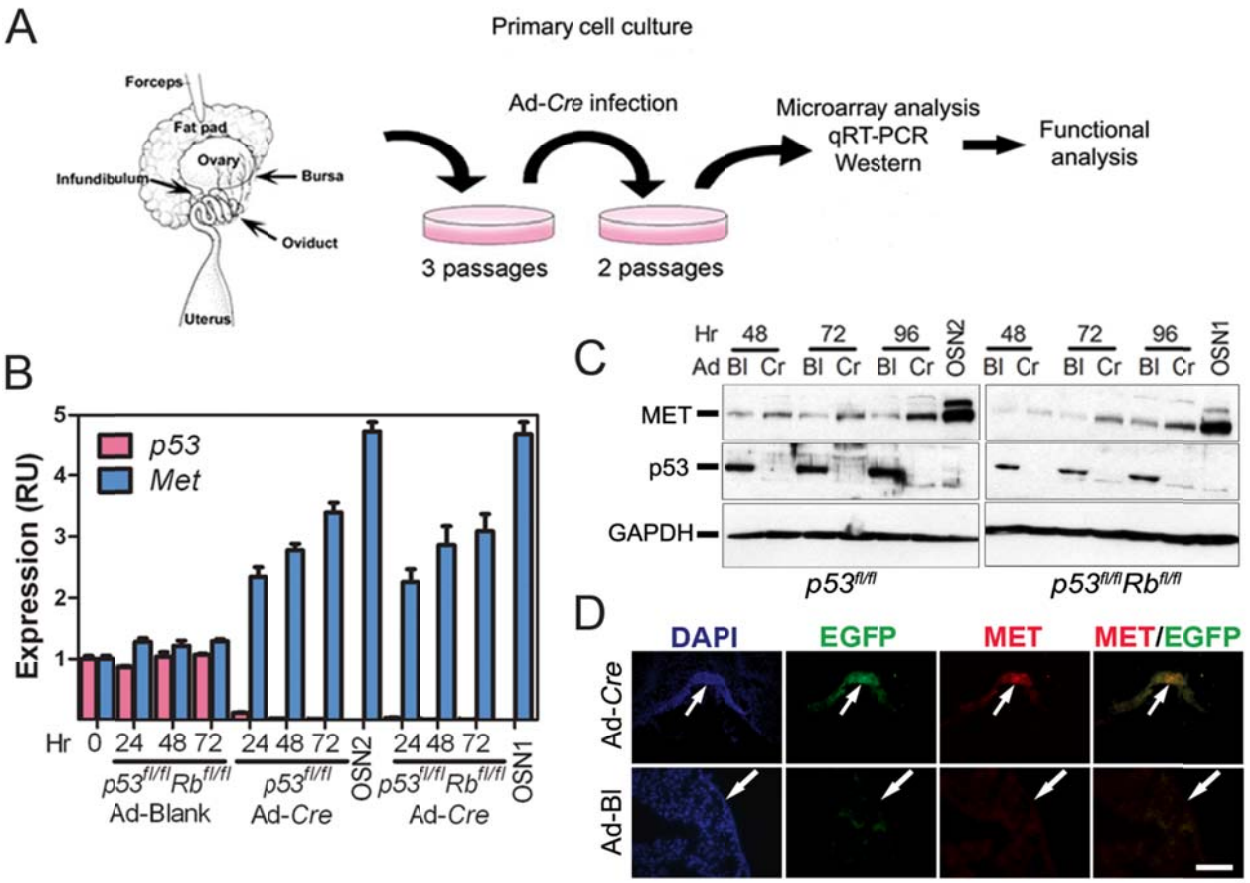
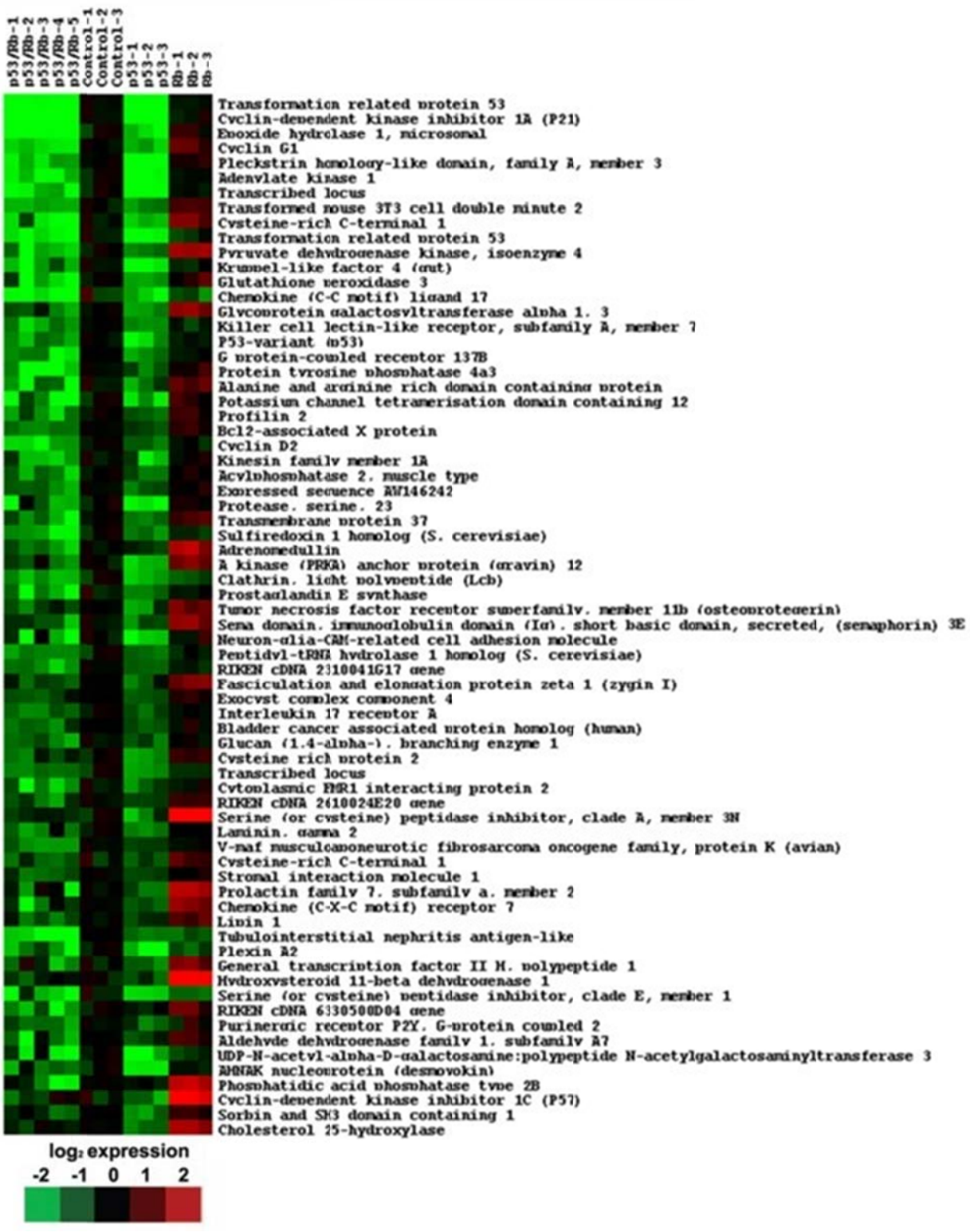
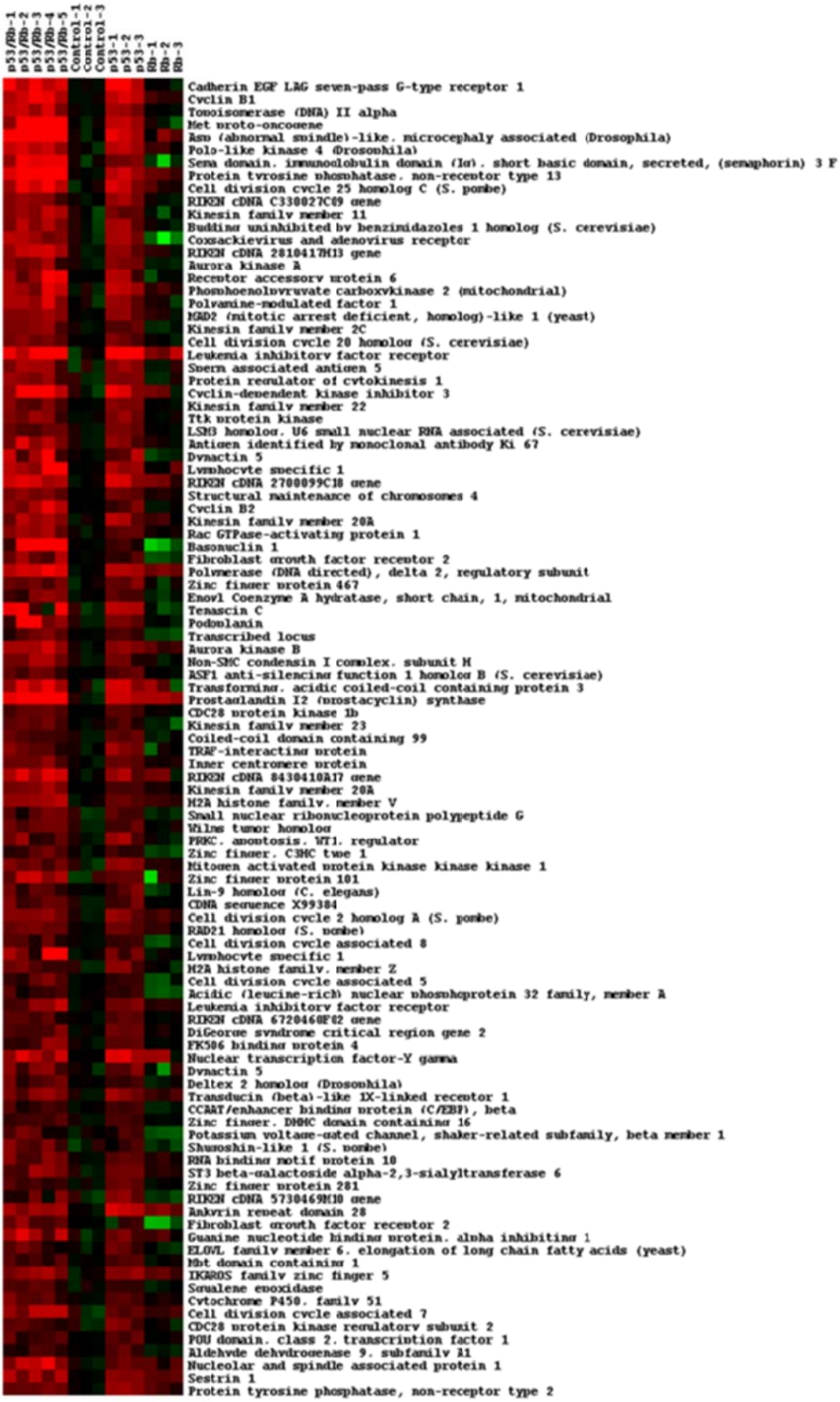

A
Primary cell culture
Forceps
Fat pad
Ovary
Infundibulum
Bursa
Oviduct
Uterus
Ad-Cre infection
3 passages
2 passages
Microarray analysis
qRT-PCR
Western
Functional analysis
B
Expression (RU)
p53
Met
Hr 0 24 48 72 24 48 72 OSN2 24 48 72 OSN1
p53fl/flRbfl/fl
Ad-Blank
p53fl/fl
Ad-Cre
p53fl/flRbfl/fl
Ad-Cre
C
Hr 48 72 96 OSN2 48 72 96 OSN1
Ad Bl Cr Bl Cr Bl Cr Bl Cr Bl Cr Bl Cr
MET
p53
GAPDH
p53fl/fl
p53fl/flRbfl/fl
D
DAPI
EGFP
MET
MET/EGFP
Ad-Cre
Ad-Bl

Figure 3.2 Gene expression profiles of *p53* inactivated OSE cells. Gene expression profiles of OSE cells from $p53^{fl/fl}$, $Rb^{fl/fl}$ and $p53^{fl/fl}Rb^{fl/fl}$ mouse were analyzed after either Ad-*LacZ* or Ad-*Cre* infection. Independent replicates were subjected to the analysis. Two class analysis (FDR < 1%) between *p53* inactivated ($p53^{fl/fl}$ and $p53^{fl/fl}Rb^{fl/fl}$ with Ad-*Cre*) and control groups ($Rb^{fl/fl}$ with Ad-*Cre* and $p53^{fl/fl}Rb^{fl/fl}$ with Ad-*LacZ*) was performed. A total of 76 and 52 genes were identified to be significantly downregulated (upper) and upregulated (lower), respectively, in the *p53* inactivated group. Among those genes, several were well established downstream targets of p53-mediated activation, such as *p21, Mdm2,* and *Bax,* and repression, such as *cyclin B1*. Kindly provided by Dr. Wei Wang, Cornell Microarray Core Facility.


p53/Rb-1
p53/Rb-2
p53/Rb-3
p53/Rb-4
p53/Rb-5
Control-1
Control-2
Control-3
p53-1
p53-2
p53-3
Rb-1
Rb-2
Rb-3
Transformation related protein 53
Cyclin-dependent kinase inhibitor 1A (P21)
Epoxide hydrolase 1, microsomal
Cyclin G1
Pleckstrin homology-like domain, family A, member 3
Adenylate kinase 1
Transcribed locus
Transformed mouse 3T3 cell double minute 2
Cysteine-rich C-terminal 1
Transformation related protein 53
Pyruvate dehydrogenase kinase, isoenzyme 4
Krumpel-like factor 4 (gut)
Glutathione peroxidase 3
Chemokine (C-C motif) ligand 17
Glycoprotein galactosyltransferase alpha 1, 3
Killer cell lectin-like receptor, subfamily A, member 7
P53-variant (p53)
G protein-coupled receptor 137B
Protein tyrosine phosphatase 4a3
Alanine and arginine rich domain containing protein
Potassium channel tetramerisation domain containing 12
Profilin 2
Bcl2-associated X protein
Cyclin D2
Kinesin family member 1A
Acylphosphatase 2, muscle type
Expressed sequence AW146242
Protease, serine, 23
Transmembrane protein 37
Sulfiredoxin 1 homolog (S. cerevisiae)
Adrenomedullin
A kinase (PRKA) anchor protein (gravin) 12
Clathrin, light polypeptide (Lcb)
Prostaglandin E synthase
Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)
Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E
Neuron-glia-CAM-related cell adhesion molecule
Peptidyl-tRNA hydrolase 1 homolog (S. cerevisiae)
RIKEN cDNA 2310041G17 gene
Fasciculation and elongation protein zeta 1 (zygin I)
Exocyst complex component 4
Interleukin 17 receptor A
Bladder cancer associated protein homolog (human)
Glucan (1,4-alpha-), branching enzyme 1
Cysteine rich protein 2
Transcribed locus
Cytoplasmic FMR1 interacting protein 2
RIKEN cDNA 2410024E20 gene
Serine (or cysteine) peptidase inhibitor, clade A, member 3N
Laminin, gamma 2
V-maf musculoaponeurotic fibrosarcoma oncogene family, protein K (avian)
Cysteine-rich C-terminal 1
Stromal interaction molecule 1
Prolactin family 7, subfamily a, member 2
Chemokine (C-X-C motif) receptor 7
Lipin 1
Tubulointerstitial nephritis antigen-like
Plexin A2
General transcription factor II H, polypeptide 1
Hydroxysteroid 11-beta dehydrogenase 1
Serine (or cysteine) peptidase inhibitor, clade E, member 1
RIKEN cDNA 6330500D04 gene
Purinergic receptor P2Y, G-protein coupled 2
Aldehyde dehydrogenase family 1, subfamily A7
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3
AHNAK nucleoprotein (desmoyokin)
Phosphatidic acid phosphatase type 2B
Cyclin-dependent kinase inhibitor 1C (P57)
Sorbin and SH3 domain containing 1
Cholesterol 25-hydroxylase
log2 expression
-2 -1 0 1 2


p53/Rb-1
p53/Rb-2
p53/Rb-3
p53/Rb-4
p53/Rb-5
Control-1
Control-2
Control-3
p53-1
p53-2
p53-3
Rb-1
Rb-2
Rb-3
Cadherin EGF LAG seven-pass G-type receptor 1
Cyclin B1
Topoisomerase (DNA) II alpha
Met proto-oncogene
Asp (abnormal spindle)-like, microcephaly associated (Drosophila)
Polo-like kinase 4 (Drosophila)
Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3 F
Protein tyrosine phosphatase, non-receptor type 13
Cell division cycle 25 homolog C (S. pombe)
RIKEN cDNA C330027C09 gene
Kinesin family member 11
Budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)
Coxsackievirus and adenovirus receptor
RIKEN cDNA 2810417H13 gene
Aurora kinase A
Receptor accessory protein 6
Phosphoenolpyruvate carboxykinase 2 (mitochondrial)
Polyamine-modulated factor 1
MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast)
Kinesin family member 2C
Cell division cycle 20 homolog (S. cerevisiae)
Leukemia inhibitory factor receptor
Sperm associated antigen 5
Protein regulator of cytokinesis 1
Cyclin-dependent kinase inhibitor 3
Kinesin family member 22
Ttk protein kinase
LSM3 homolog, U6 small nuclear RNA associated (S. cerevisiae)
Antigen identified by monoclonal antibody Ki 67
Dynactin 5
Lymphocyte specific 1
RIKEN cDNA 2700099C18 gene
Structural maintenance of chromosomes 4
Cyclin B2
Kinesin family member 20A
Rac GTPase-activating protein 1
Basonuclin 1
Fibroblast growth factor receptor 2
Polymerase (DNA directed), delta 2, regulatory subunit
Zinc finger protein 467
Enoyl Coenzyme A hydratase, short chain, 1, mitochondrial
Tenascin C
Podoplanin
Transcribed locus
Aurora kinase B
Non-SMC condensin I complex, subunit H
ASF1 anti-silencing function 1 homolog B (S. cerevisiae)
Transforming, acidic coiled-coil containing protein 3
Prostaglandin I2 (prostacyclin) synthase
CDC28 protein kinase 1b
Kinesin family member 23
Coiled-coil domain containing 99
TRAF-interacting protein
Inner centromere protein
RIKEN cDNA 8430410A17 gene
Kinesin family member 20A
H2A histone family, member V
Small nuclear ribonucleoprotein polypeptide G
Wilms tumor homolog
PRKC, apoptosis, WT1, regulator
Zinc finger, C3HC type 1
Mitogen activated protein kinase kinase kinase 1
Zinc finger protein 101
Lin-9 homolog (C. elegans)
cDNA sequence X99384
Cell division cycle 2 homolog A (S. pombe)
RAD21 homolog (S. pombe)
Cell division cycle associated 8
Lymphocyte specific 1
H2A histone family, member Z
Cell division cycle associated 5
Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
Leukemia inhibitory factor receptor
RIKEN cDNA 6720460F02 gene
DiGeorge syndrome critical region gene 2
FK506 binding protein 4
Nuclear transcription factor-Y gamma
Dynactin 5
Deltex 2 homolog (Drosophila)
Transducin (beta)-like 1X-linked receptor 1
CCAAT/enhancer binding protein (C/EBP), beta
Zinc finger, DHHC domain containing 16
Potassium voltage-gated channel, shaker-related subfamily, beta member 1
Shugoshin-like 1 (S. pombe)
RNA binding motif protein 10
ST3 beta-galactoside alpha-2,3-sialyltransferase 6
Zinc finger protein 281
RIKEN cDNA 5730469M10 gene
Ankyrin repeat domain 28
Fibroblast growth factor receptor 2
Guanine nucleotide binding protein, alpha inhibiting 1
ELOVL family member 6, elongation of long chain fatty acids (yeast)
Mbt domain containing 1
IKAROS family zinc finger 5
Squalene epoxidase
Cytochrome P450, family 51
Cell division cycle associated 7
CDC28 protein kinase regulatory subunit 2
POU domain, class 2, transcription factor 1
Aldehyde dehydrogenase 9, subfamily A1
Nucleolar and spindle associated protein 1
Sestrin 1
Protein tyrosine phosphatase, non-receptor type 2

HCT116 carrying wild-type p53 resulted in increased MET expression (Figure 3.3A). To examine whether MET overexpression would be detected in vivo early after *p53* inactivation, Ad-*Cre* was delivered to the OSE of $p53^{fl/fl}Rb^{fl/fl}$ Z/EG mice by transoviductal injection. Consistent with the cell culture experiments, elevated levels of MET were detected in OSE cells that had Cre-*loxP* mediated recombination according to expression of EGFP reporter 72 hours after Ad-*Cre* administration, but Ad-blank administration did not result in detectable MET or EGFP expression (Figure 3.1D). No morphologically detectable differences were observed between mutant and wild-type OSE at that time (Figure 3.3B).

3.4.2 MET is essential for p53-controlled cell motility and invasion.

Because increased motility and invasion are among the principal effects of upregulated MET signaling, those features were tested in p53-deficient OSE cells. Compared to cells with wild-type p53, p53 null cells showed significantly increased cell motility in wound healing/time-lapse microscopy and migration assays as well as increased propensity for invasion in Matrigel chambers (Figure 3.4A-D, Figure 3.5A). Moreover, treatment of cells with MET ligand HGF enhanced migration and invasion even further (Figure 3.4A-B). The increased motility and invasion was accompanied by elevation of levels of phosphorylated MET (Figure 3.5B). To test the extent of MET contributions to these properties of p53 null cells, both genes were inactivated in OSE cells derived from $p53^{fl/fl}Met^{fl/fl}$ mice. *Met* inactivation abrogated the motility and invasion, but not proliferation phenotype associated with *p53* inactivation (Figure 3.4E and Figure 3.6). Thus, MET signaling is essential for p53-controlled motility and invasion.

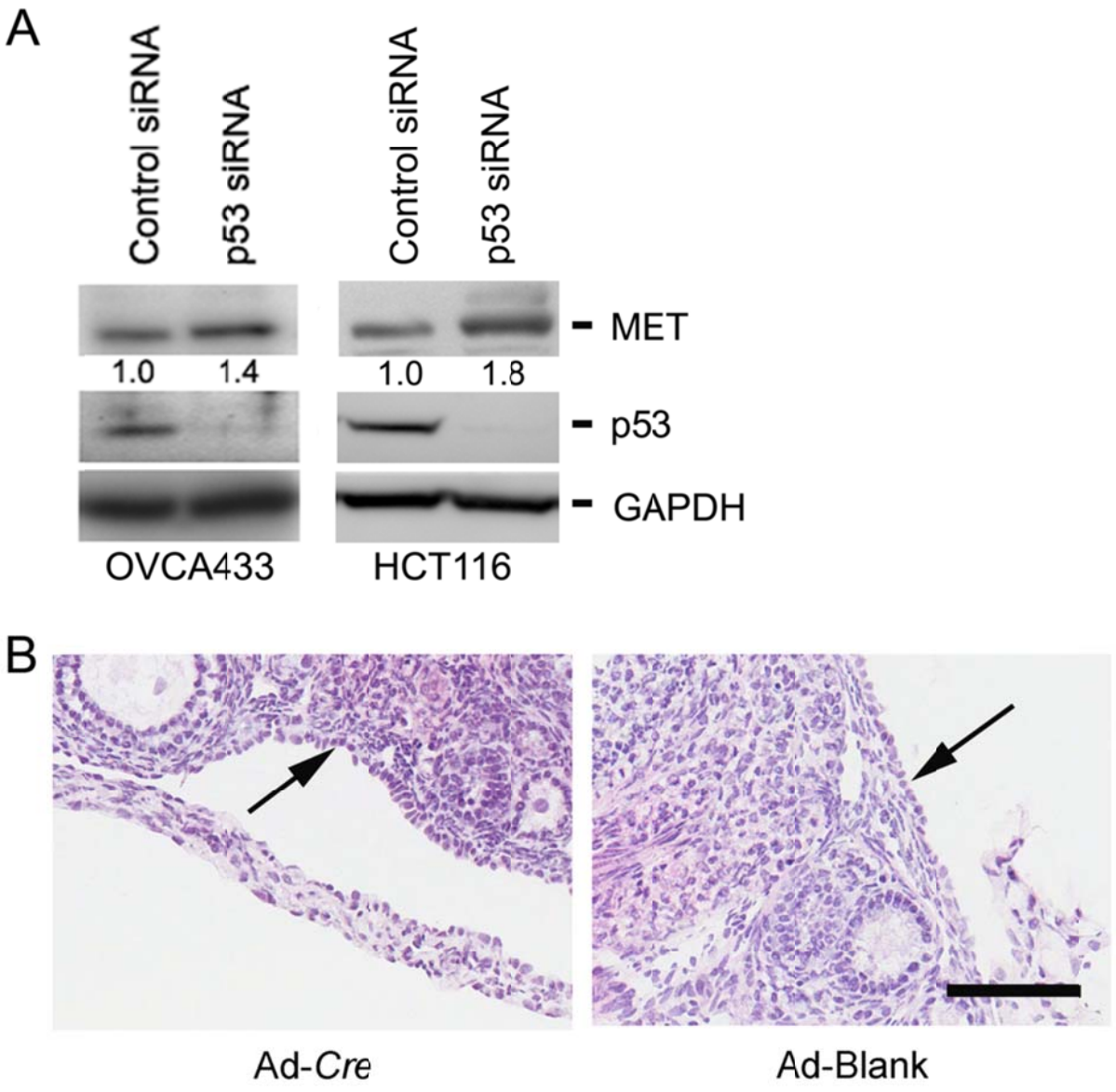
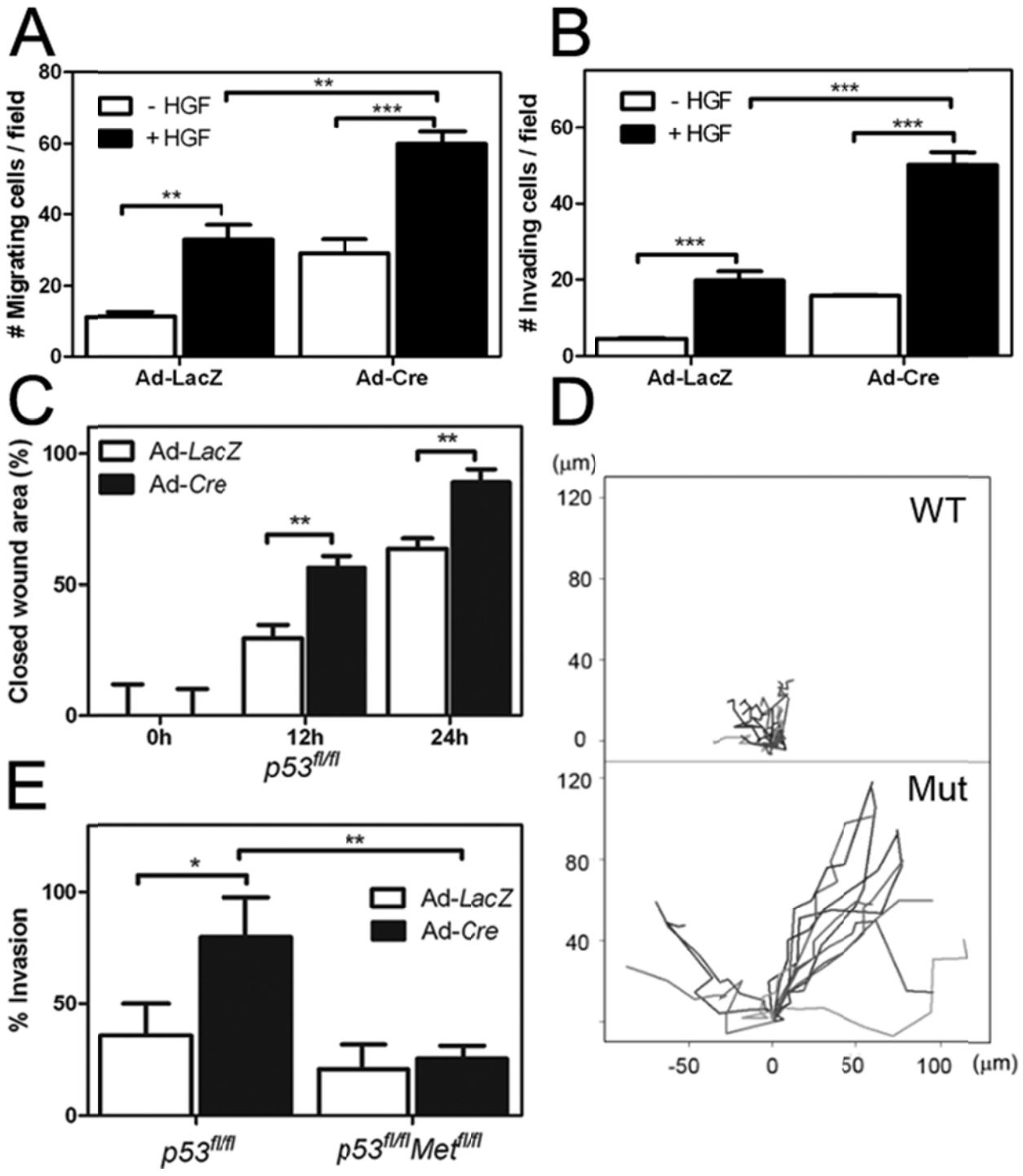


Figure 3.3 Effect of p53 knockdown on MET expression in human cancer cells expressing wild-type p53 and OSE morphology. (A) 20 nM of either control or p53 siRNA was transfected into OVCA433 and HCT116 cells and MET expression was measured by Western blotting 48 hrs afterward. (B) OSE (arrow) 72 hrs after either Ad-*Cre* (left) or Ad-Blank (right) infection. H&E staining. Scale bar, 100 μm.

Figure 3.4 MET is essential for p53 inactivation-mediated cell motility and invasion. (A and B) Cell migration and invasion 24 hours after *p53* inactivation either in absence or presence of 20 ng/ml HGF in the lower chamber. (C) Motility of primary $p53^{fl/fl}$ OSE cells 72 hrs after infection with either Ad-*LacZ* or Ad-*Cre*. Percentages (Mean ± SD) of closed wound area 0, 12 and 24 hrs after the scratch were measured by TScratch software. (D) Tracking of individual wild-type (WT) and null *p53* (Mut) cells (n=14 each) in wound healing assay using time-lapse microscopy followed by analysis with ImageJ multi-track software. (E) Invasion properties of primary OSE cells isolated from $p53^{fl/fl}$ and $p53^{fl/fl}Met^{fl/fl}$ mice. Cells (2.5×10^4) were seeded into either control or Matrigel inserts 24 hrs after Ad-*LacZ* or Ad-*Cre* infection with HGF (20 ng/ml) in the lower chamber. Invading cells were counted after staining under the microscope 20 hrs afterwards. Bars, SD (n=3). *p<0.05; **p<0.01; ***p<0.001.


A
Migrating cells / field
- HGF
+ HGF
Ad-LacZ
Ad-Cre
B
Invading cells / field
C
Closed wound area (%)
Ad-LacZ
Ad-Cre
0h
12h
24h
p53fl/fl
D
(μm)
WT
Mut
E
% Invasion
p53fl/fl
p53fl/flMetfl/fl

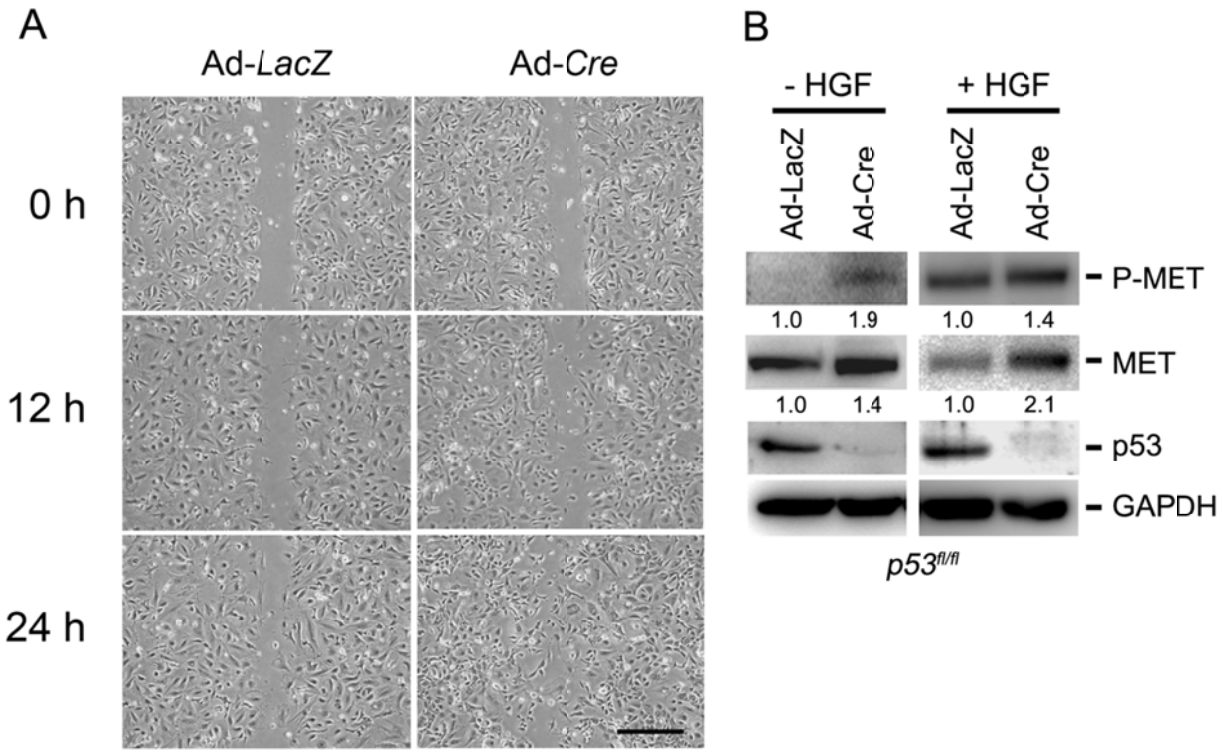
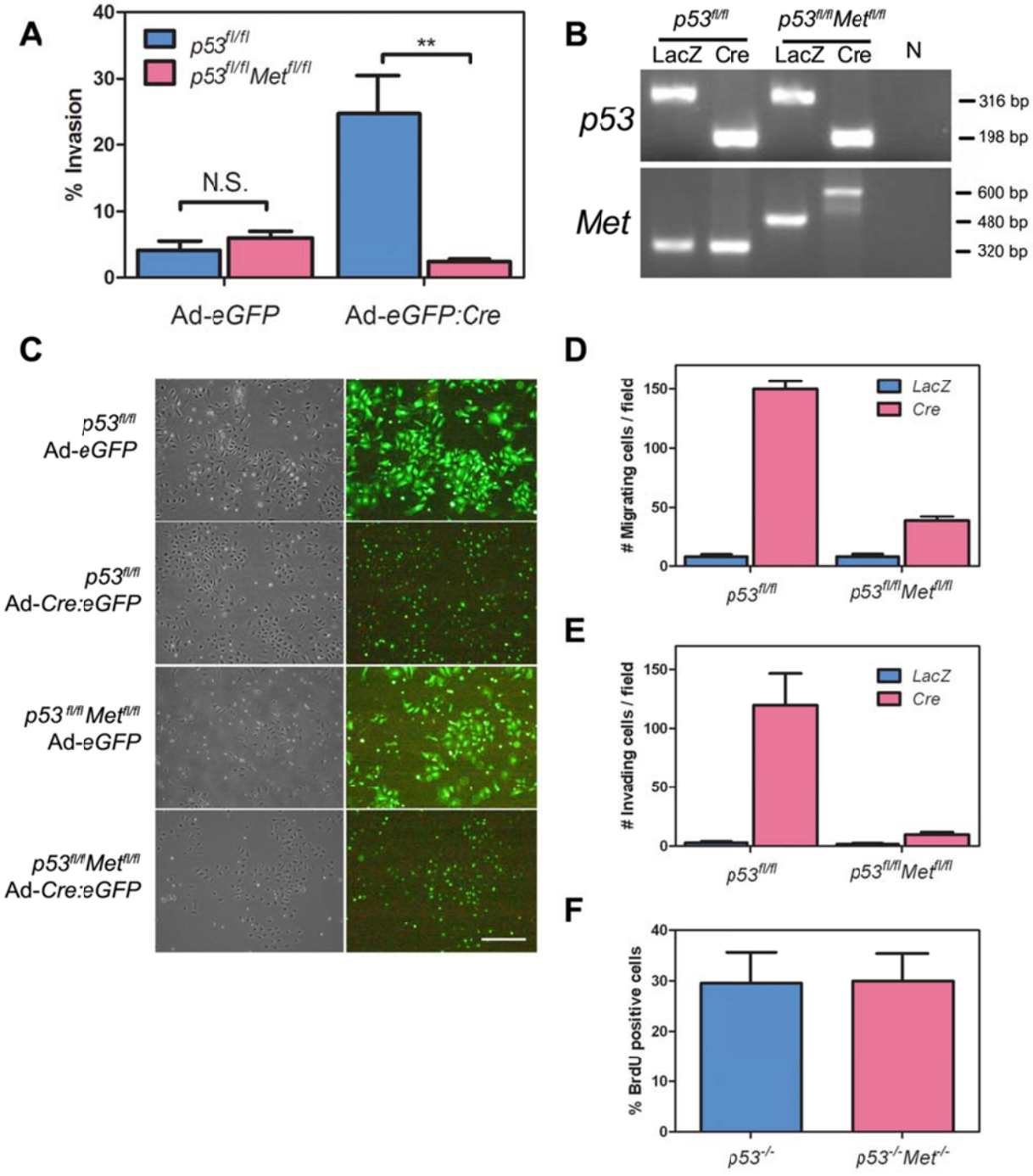


Figure 3.5 *p53* inactivation increases cell motility along with increased MET expression and MET phosphorylation. (A) Either Ad-*LacZ* or Ad-*Cre* infected OSE cells from *p53*$^{fl/fl}$ mouse were cultured, and then scratch was made by a p200 tip. Wound healing was monitored in 12 hrs and 24 hrs. Quantitative measurements of the assay are presented in Figure 3.4C. Scale bar, 500 μm. (B) 24 hrs after either Ad-*LacZ* or Ad-*Cre* infection, OSE cells from *p53*$^{fl/fl}$ mouse were treated with either blank solution (-HGF) or 20 ng/ml HGF (+HGF) and MET and phospho-MET expression were measured by Western blotting after another 24 hrs in cell culture.

Figure 3.6 Migration and invasion but not proliferation depends on MET presence after *p53* inactivation. (A) Percentage of invasion was measured in OSE cells by using either Ad-*EGFP* or Ad-*Cre:EGFP*. (B) Cre-*loxP* mediated recombination was confirmed by PCR. Floxed and recombined *p53* allele were detected as 316 bp and 198 bp DNA fragments respectively. Wild-type, floxed and recombined *Met* allele were detected as 480 bp, 320 bp and 600 bp fragments, respectively. (C) Adenoviral infection efficiency was monitored by fluorescent microscopy one day after infection. Over 90% of cells showed strong green fluorescence. Scale bar, 500 μm. (D-E) Either Ad-*LacZ* or Ad-*Cre* infected OSE cells from $p53^{fl/fl}$ and $p53^{fl/fl}Met^{fl/fl}$ mice were seeded onto either empty or Matrigel insert for counting migrating (D) or invading (E) cells, respectively, in the presence of HGF (20 ng/ml). (F) OSE cells from either $p53^{fl/fl}$ or $p53^{fl/fl}Met^{fl/fl}$ mice were incubated with BrdU for 2 hrs 48 hrs after infection with Ad-*Cre*. BrdU positive cells were detected by anti-BrdU antibody and counted. Bar, SD, (n=3) ; N.S., not significant ; **p<0.01.


A
% Invasion
p53fl/fl
p53fl/flMetfl/fl
N.S.
**
Ad-eGFP
Ad-eGFP:Cre
B
p53fl/fl
p53fl/flMetfl/fl
LacZ Cre
LacZ Cre
N
p53
Met
316 bp
198 bp
600 bp
480 bp
320 bp
C
p53fl/fl Ad-eGFP
p53fl/fl Ad-Cre:eGFP
p53fl/flMetfl/fl Ad-eGFP
p53fl/flMetfl/fl Ad-Cre:eGFP
D
Migrating cells / field
LacZ
Cre
p53fl/fl
p53fl/flMetfl/fl
E
Invading cells / field
LacZ
Cre
p53fl/fl
p53fl/flMetfl/fl
F
% BrdU positive cells
p53-/-
p53-/-Met-/-

3.4.3 p53 has a miR-34-independent mechanism of MET regulation.

Consistent with regulation of MET expression by p53, expression of p53 at levels comparable to those of endogenous activation resulted in decreased amounts of MET protein in both mouse and human neoplastic ovarian cell lines, OSN2 and SKOV-3, respectively (Figure 3.7A and Figure 3.8).

To test the extent of MET expression dependence on miR-34, ovarian neoplastic cells were subjected to increasing amounts of miR-34 precursor molecules in transient transfection experiments. Maximum reduction of MET expression was observed at 30 nM concentration with no apparent effect of further increases in amounts of miR-34a precursor molecule (Figure 3.9A-B) or combination of miR-34a, b and c (Corney et al., 2010). No effect on *MET* promoter activity was observed after transfection of the full length *MET* promoter reporter construct (pGL2-3.1MET) together with individual miR-34 family precursor molecules (Figure 3.9C). These observations were in agreement with bioinformatics predictions and experimental evidences that miR-34 regulates MET expression principally by targeting MET 3'-UTR (reviewed in (Hermeking, 2007)). However co-transfection of miR-34a precursor together with wild-type p53, resulted in further downregulation of MET (Figure 3.7A), suggesting that p53 may have a miR-34-independent mechanism of MET regulation. Similar effects of p53 and/or miR-34a precursor transfection on MET downregulation were observed in p53 null lung cancer cells NCI-H1299, indicating potential significance of these observations for pathogenesis of other types of epithelial cancers (Figure 3.9D).

To directly show the presence of miR-34-independent mechanism of MET regulation by p53, we isolated OSE cells from *mir-34a*$^{-/-}$*mir-34b/c*$^{-/-}$ (triple knockout, TKO) mice, lacking the entire *mir-34* family of genes. As expected, OSE cells from TKO mice showed higher level of

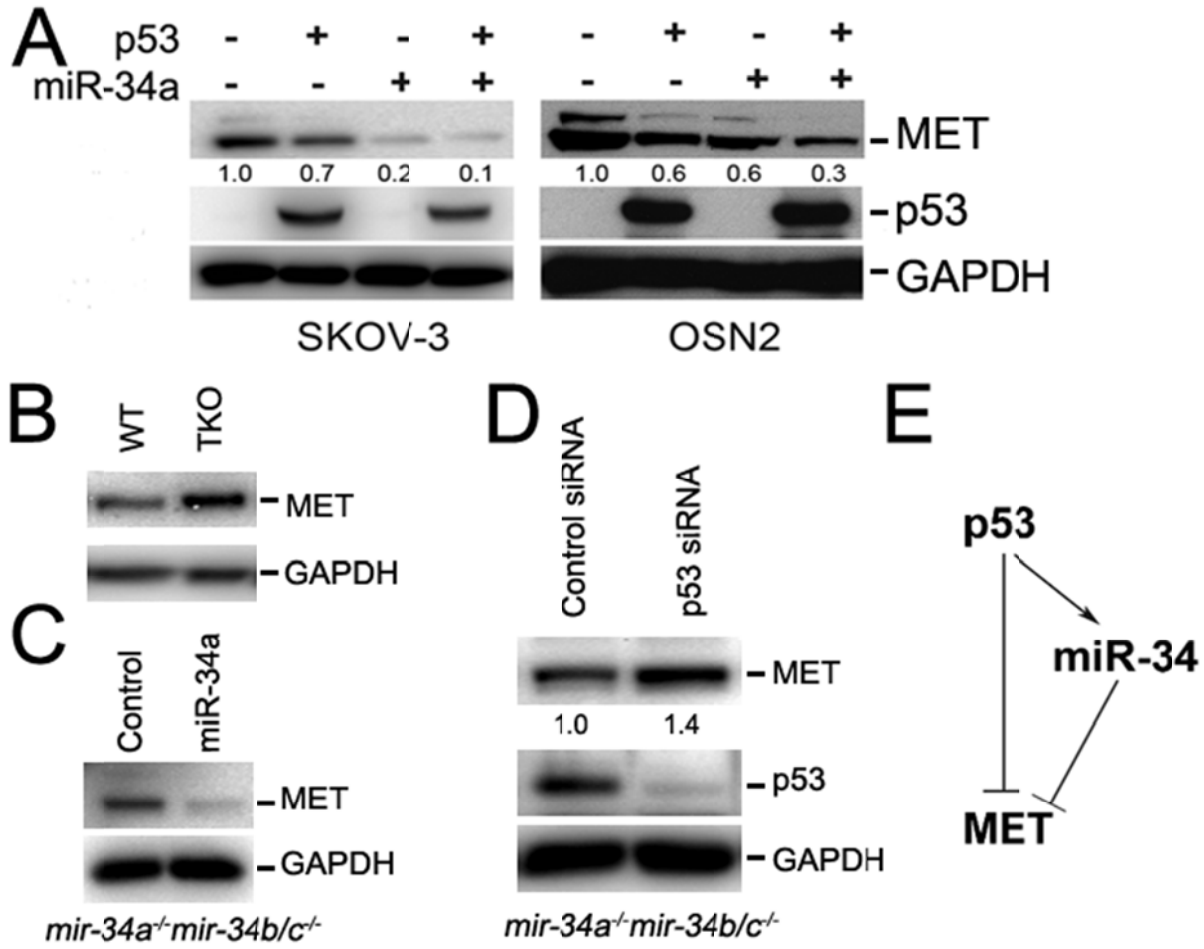


Figure 3.7 p53 has a miR-34-independent mechanism of MET regulation. (A) MET expression after transient transfection of p53 expression vector (pORF-hp53) and/or miR-34a precursor molecule (30 nM) into SKOV-3 or OSN2 cells. (B) MET expression in OSE cells derived from either *mir-34a*$^{-/-}$*mir-34b/c*$^{-/-}$ (TKO) or age-matched wild-type (WT) mice. (C) MET expression in TKO OSE cells after transfection with either scrambled microRNA (control) or 30 nM miR-34a precursor molecules. (D) MET expression in TKO OSE cells 48 hrs after transfection with either control siRNA or p53 siRNA (20 nM). (E) A diagram of proposed miR-34 dependent and independent regulation of MET by p53.

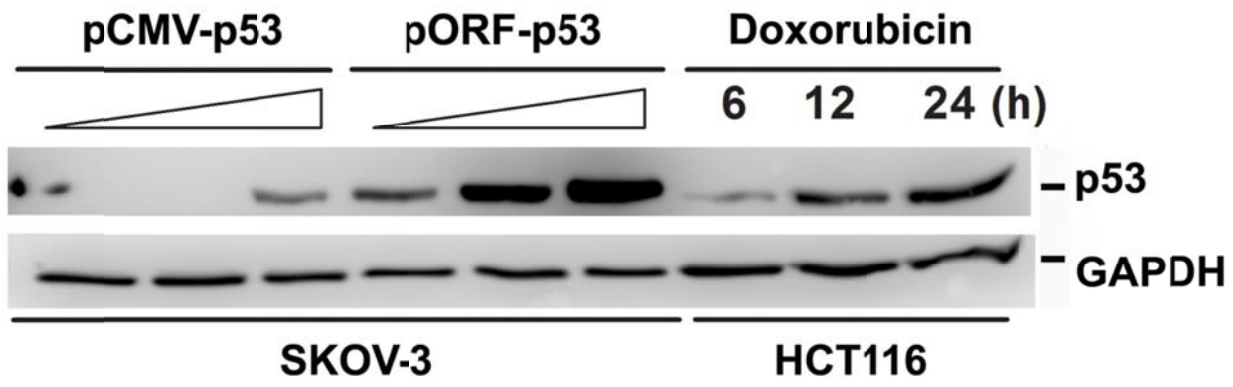
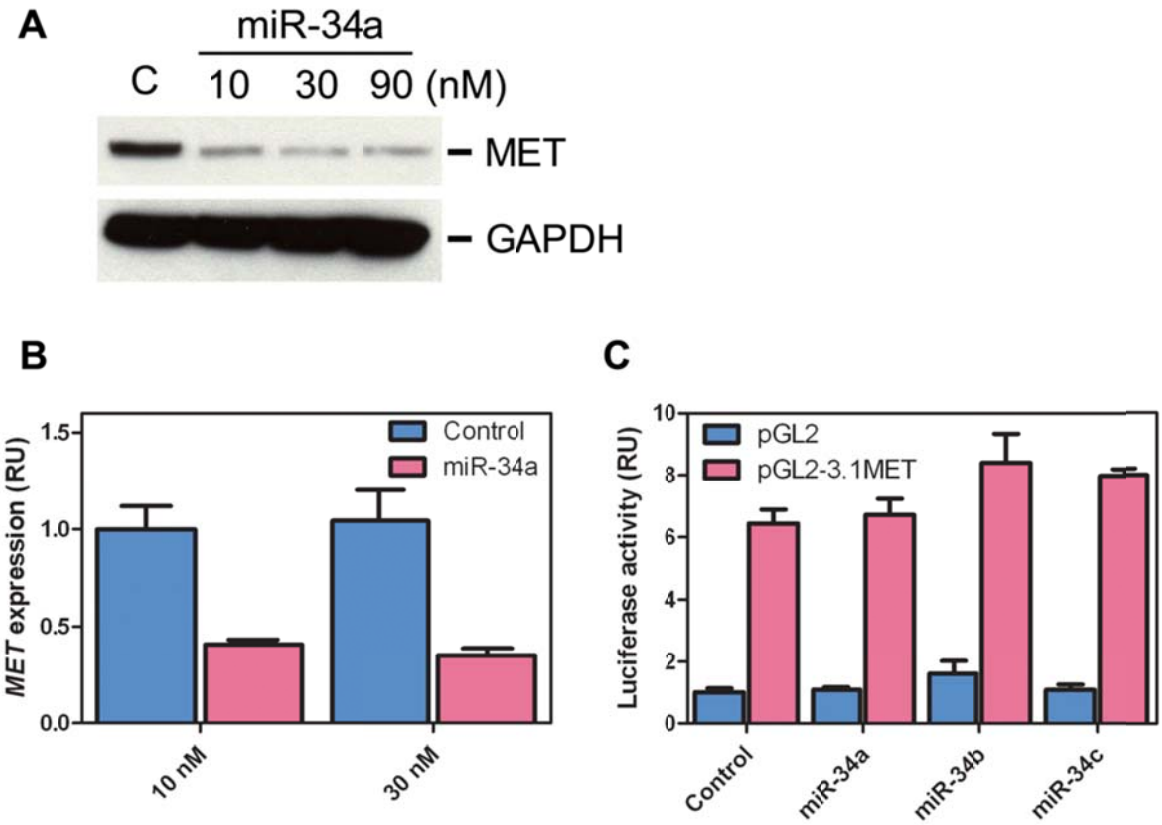
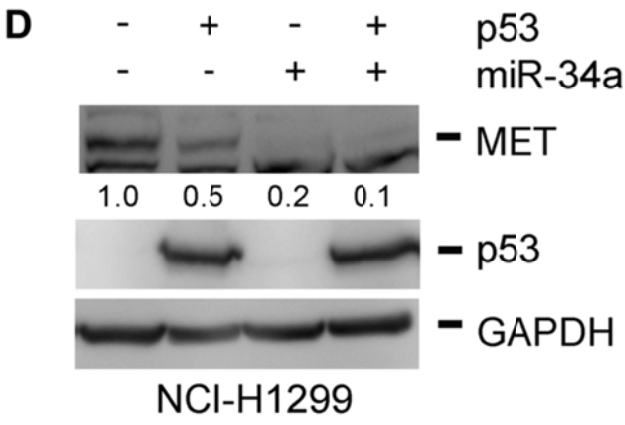


Figure 3.8 Ectopic p53 expression is comparable to endogenous p53 expression level. pCMV-p53 and pORF-hp53 were transfected into SKOV-3. In the case of pCMV-p53, the highest concentration was used for all promoter analysis. In the case of pORF-p53, the lowest concentration or lesser amount was used. In order to compare endogenous p53 level, wild-type p53 harboring HCT116 cells were treated with doxorubicin (2 μg/ml) for 6, 12 or 24 hrs.

Figure 3.9 miR-34 and p53 effects on MET expression and promoter activity. (A-B) miR-34a precursor molecules were transfected into SKOV-3 cells at 10 nM, 30 nM or 90 nM concentration. Note, that miR-34a transfection at 30 nM is sufficient for the maximum effect on MET downregulation. MET protein and mRNA level were measured by Western blot (A) and qRT-PCR (B) analysis. Bars, SD. n=3. **P<0.01. (C) The effect of miR-34 family on *MET* promoter activity. Individual *miR-34* family members and pGL2-3.1MET promoter construct were co-transfected into SKOV-3 cells. Luciferase activity was measured 24 hrs after transfection. Bars, SD. n=3. (D) MET expression after transient transfection of p53 expression vector (pORF-hp53) and/or miR-34a precursor molecule (30 nM) into NCI-H1299 cells.


A
miR-34a
C
10
30
90 (nM)
MET
GAPDH
B
MET expression (RU)
1.5
1.0
0.5
0.0
Control
miR-34a
10 nM
30 nM
C
Luciferase activity (RU)
10
8
6
4
2
0
pGL2
pGL2-3.1MET
Control
miR-34a
miR-34b
miR-34c


D
-
+
-
+
p53
-
-
+
+
miR-34a
MET
1.0
0.5
0.2
0.1
p53
GAPDH
NCI-H1299

MET compared with wild-type OSE cells (Figure 3.7B). Reconstitution of miR-34 by transfecting miR-34a precursor molecule resulted in downregulation of MET (Figure 3.7C). Notably, p53 knockdown in TKO OSE cells led to an increase of MET expression (Figure 3.7D), confirming that p53 down-regulates MET expression in a miR-34-independent manner, in addition to miR-34-dependent mechanism (Figure 3.7E).

3.4.4 p53 suppresses MET promoter activity.

To test if *MET* promoter activity can be affected by p53 through binding of p53 consensus sequence in ovarian cells, reporter constructs containing -2619 to +353 fragment of *MET* upstream sequence (3.1MET) and its shorter fragments (Gambarotta et al., 1996) were co-transfected with p53 expression vector into SKOV-3 (Figure 3.10) and OSN2 cells (Figure 3.11A-B). Transfection of p53 significantly suppressed the promoter activity of 3.1MET construct, as well as that of smaller fragments. Even when the putative p53 responsive element was mutated (0.65METm), p53 was still capable of repressing *MET* promoter activity comparably to the repression by corresponding wild-type promoter construct (0.65MET), indicating that the discrepancy between previous report (Seol et al., 1999) and our findings may be a result of cell type-specific effects.

To rule out the possibility that the observed effects of p53 on *MET* promoter activity were caused by global transcriptional repression by ectopically expressed p53, PG-13-luc and MG-15-luc reporter constructs, containing 13 copies of wild-type p53 consensus sequence, and 15 copies of slightly mutated p53 consensus sequence (El-Deiry et al., 1993), respectively, were co-transfected with plasmids expressing either wild-type or DNA binding domain mutant p53. Only wild-type p53 could activate PG-13-luc promoter activity in SKOV3 (Figure 3.11C) and

Figure 3.10 p53 suppresses *MET* promoter activity. (A) Promoter constructs used in this study. 3.1 Kb human *MET* promoter sequence including 5'-UTR region (3.1MET) and its shorter fragments were cloned into pGL2-luciferase vector. p53 putative response element (star) was mutated in 0.65METm. (B-C) *MET* promoter activity after co-transfection of p53 expression vector (pORF-hp53) with individual promoter constructs into SKOV-3 cells. Cell lysates were harvested for estimation of luciferase activity 48 hrs after transfection. Note that p53 response element mutation does not abolish *MET* promoter suppression by p53 (C). All experiments were performed in triplicates. Bars, SD.

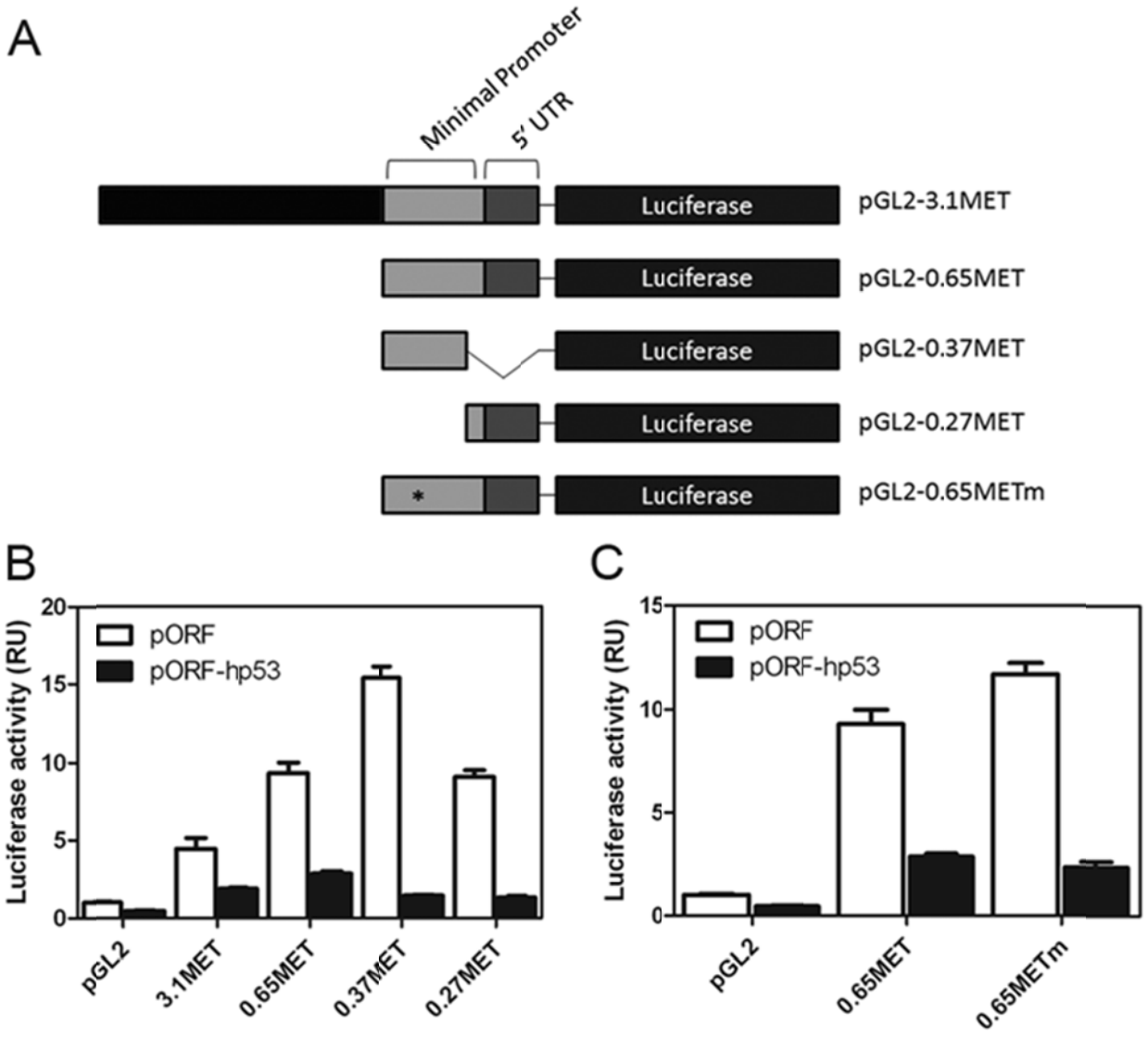
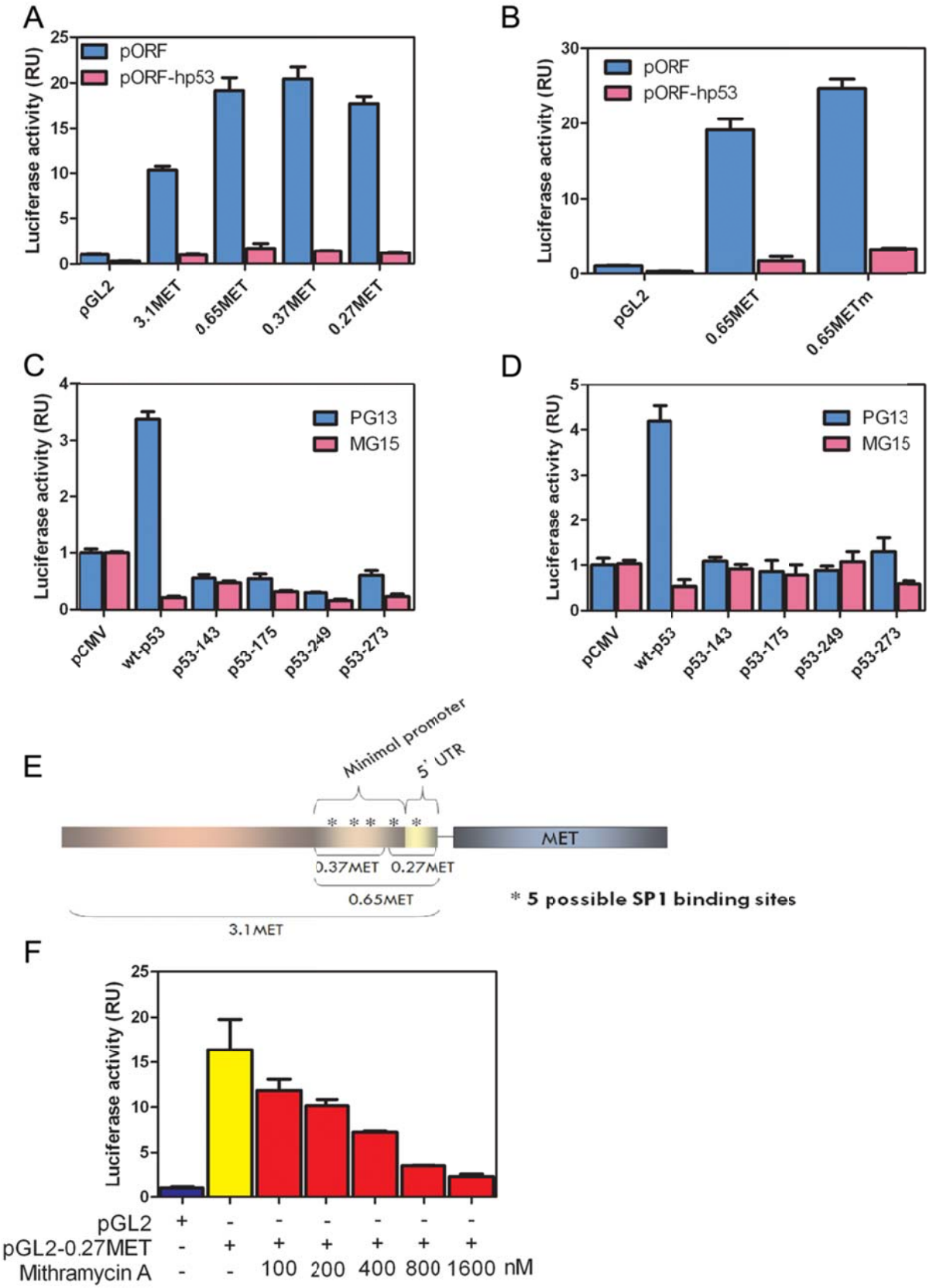

A
Minimal Promoter
5′ UTR
Luciferase
pGL2-3.1MET
Luciferase
pGL2-0.65MET
Luciferase
pGL2-0.37MET
Luciferase
pGL2-0.27MET
*
Luciferase
pGL2-0.65METm
B
Luciferase activity (RU)
20
15
10
5
0
pORF
pORF-hp53
pGL2
3.1MET
0.65MET
0.37MET
0.27MET
C
Luciferase activity (RU)
15
10
5
0
pORF
pORF-hp53
pGL2
0.65MET
0.65METm

Figure 3.11 *MET* promoter activity in OSN2 cells and involvement of SP1 in *MET* promoter activity. (A) p53 expression vector (pORF-hp53) and each promoter construct were co-transfected in OSN2 cells. Cell lysates were harvested and luciferase activity was measured 24 hr after transfection. (B) p53 responsive element mutation does not abolish *MET* promoter suppression by p53. (C-D) PG13-luc, which contains 13 copies of p53 responsive elements, was co-transfected with pCMV, pCMV-wild-type p53 or pCMV-mutant p53 (V143A, R175H, R249S, and R273H) into SKOV-3 (C) and OSN2 (D) cell lines. MG15-luc, which contains 15 copies of mutated p53 responsive elements, was co-transfected with pCMV, pCMV-wild-type p53 or pCMV-mutant p53 (V143A, R175H, R249S and R273H) in SKOV-3 (C) and OSN2 (D) cell line. (E) In web-based TF binding sites algorithm with 85 threshold score (default setting), 5 potential SP1 binding sites were identified in both pGL2-0.37MET and pGL2-0.27MET. (F) OSN2 cells were pretreated with different doses of mithramycin A one hr before transfection with 0.27MET promoter construct. Every experiment was repeated 3 times. Bars, SD.


A
Luciferase activity (RU)
pORF
pORF-hp53
pGL2
3.1MET
0.65MET
0.37MET
0.27MET
B
Luciferase activity (RU)
pORF
pORF-hp53
pGL2
0.65MET
0.65METm
C
Luciferase activity (RU)
PG13
MG15
pCMV
wt-p53
p53-143
p53-175
p53-249
p53-273
D
Luciferase activity (RU)
PG13
MG15
pCMV
wt-p53
p53-143
p53-175
p53-249
p53-273
E
Minimal promoter
5' UTR
MET
0.37MET
0.27MET
0.65MET
3.1MET
* 5 possible SP1 binding sites
F
Luciferase activity (RU)
pGL2
pGL2-0.27MET
Mithramycin A
100 200 400 800 1600 nM

OSN2 cells (Figure 3.11D); all four mutant p53 constructs were unable to activate both promoter constructs.

Furthermore, p53 has been shown to inhibit hypoxia-inducible factor-stimulated transcription by destabilizing hypoxia-inducible factor 1 alpha (HIF1A), one of the prominent regulators of *MET* promoter activity (Pennacchietti et al., 2003). However, *p53* inactivation did not lead to accumulation of HIF1A in OSE cells (Figure 3.12A), likely because of uneven sensitivity of different cell types to hypoxia (Chi et al., 2006). Consistent with cell type-specificity, OSE cells and ovarian cancer cell lines (OVCA432, OVCA433 and OVCAR-3) did not overexpress MET under hypoxic conditions regardless of HIF1A accumulation (Figure 3.12B), unlike colon cancer HCT-116 cells (Figure 3.12C-D).

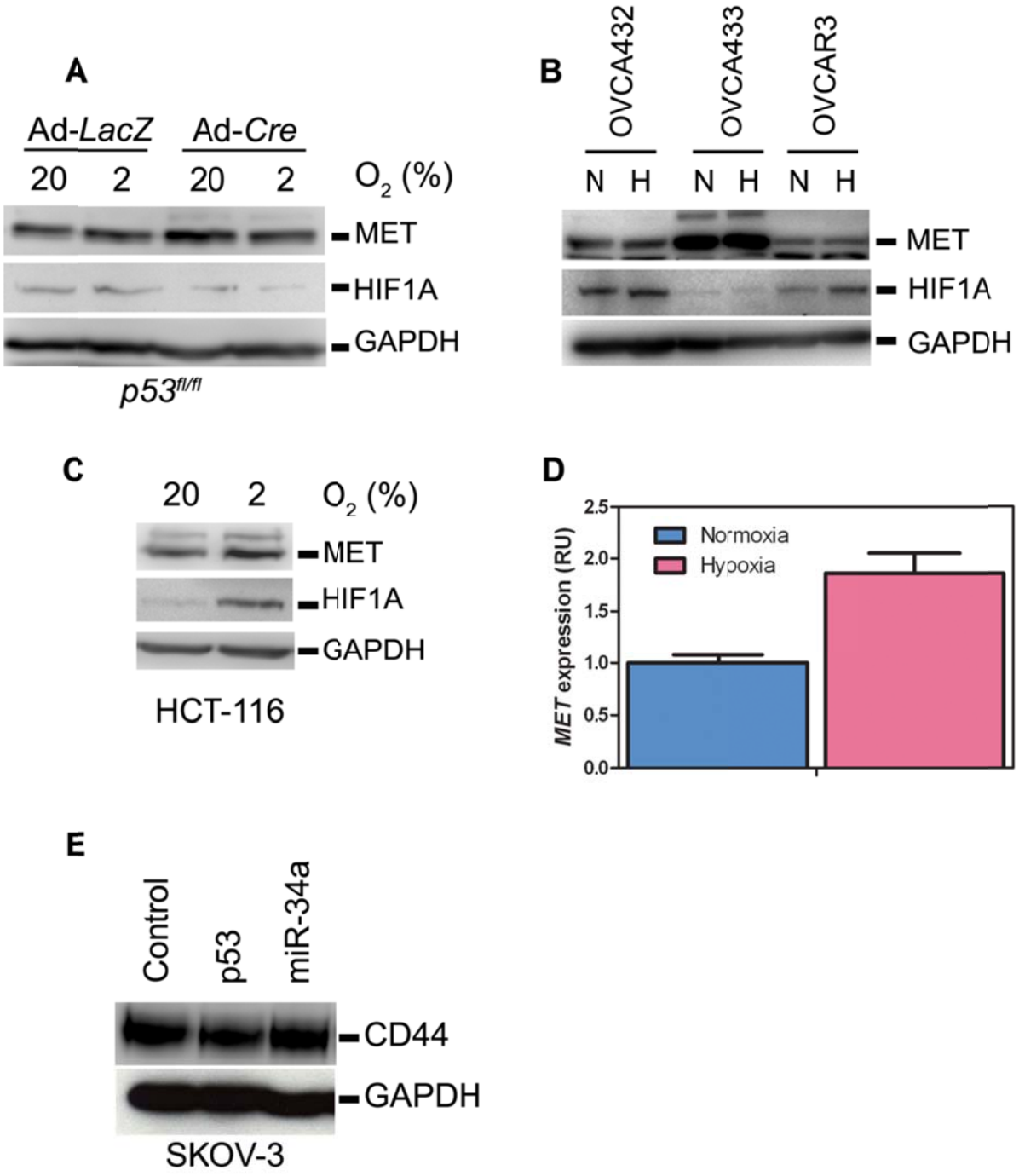
Another potential mechanism for p53-dependent regulation of MET is through CD44. CD44 is known to form a multimeric complex and cooperate with MET (Orian-Rousseau et al., 2002). It has been also recently identified as a p53 (Godar et al., 2008) and miR-34 (Liu et al., 2011) target. However, CD44 was not among genes significantly upregulated after p53 inactivation in gene expression microarrays (Figure 3.2). Furthermore, ectopic p53 or miR-34 expression did not affect CD44 levels in ovarian carcinoma cells (Figure 3.12E).

Taken together, these results show that MET suppression by p53 is unlikely to be the result of global transcriptional repression, HIF1A, or CD44 expression.

3.4.5 p53 inhibits SP1 binding to MET promoter.

Because p53 was able to suppress activity of non-overlapping promoter constructs 0.27MET and 0.37MET, transcription factor binding sites common for both constructs were searched for

Figure 3.12 Hypoxia does not affect HIF1A and MET expression, and CD44 is not affected by p53 expression in OSE cells. (A) *p53*$^{fl/fl}$ OSE cells were exposed for 24 hr to either normoxic (20% O_2) or hypoxic (2% O_2) conditions 24 hrs after exposure to Ad-*LacZ* or Ad-*Cre*. MET and HIF1A were detected by western blotting. (B) Three human ovarian cancer cell lines were exposed for 24 hrs to either normoxic (N ; 20% O_2) or hypoxic (H ; 2% O_2) conditions. (C-D). HIF1A and MET expression in HCT-116 cell line exposed to either normoxic or hypoxic conditions. Western blotting (C) and quantitative RT-PCR (D). n=3 ; Bars, SD. (E) CD44 protein levels after pORF-p53 and miR-34a precursor molecule transfection into SKOV-3 cells.

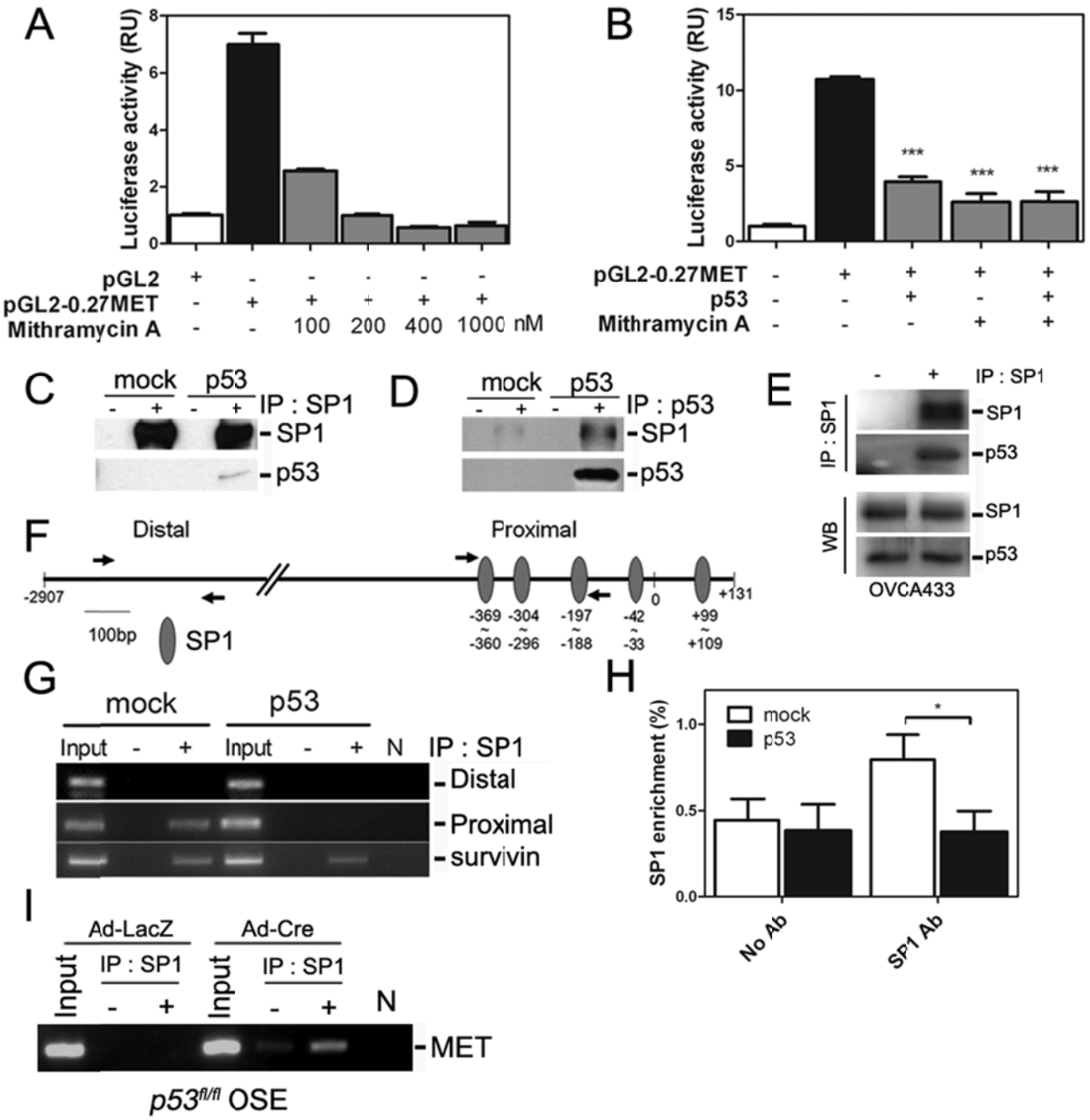

A
Ad-LacZ
Ad-Cre
20
2
20
2
O2 (%)
MET
HIF1A
GAPDH
p53fl/fl
B
OVCA432
OVCA433
OVCAR3
N
H
N
H
N
H
MET
HIF1A
GAPDH
C
20
2
O2 (%)
MET
HIF1A
GAPDH
HCT-116
D
MET expression (RU)
2.5
2.0
1.5
1.0
0.5
0.0
Normoxia
Hypoxia
E
Control
p53
miR-34a
CD44
GAPDH
SKOV-3

by bioinformatics analysis. This search identified binding sites for the SP1 transcription factor (Figure 3.11E). To test whether SP1 is required for *MET* promoter activity, SKOV-3 and OSN2 cells were treated with mithramycin A (mitA) to inhibit DNA binding of SP1 (Blume et al., 1991). MitA treatment suppressed luciferase expression under control of *MET* promoter in a dose-dependent manner, indicating that SP1 is required for *MET* promoter activity (Figure 3.13A and 3.11F). This suppression was not increased by p53 expression, indicating that p53 and SP1 share a common molecular pathway to regulate *MET* promoter activity (Figure 3.13B).

Coimmunoprecipitation experiments demonstrated that either ectopic or endogenous p53 physically interacts with endogenous SP1 in SKOV-3 and OVCA433 cells, respectively (Figure 3.13C-E). To test if p53 may inhibit SP1 DNA binding ability, ChIP experiments were performed with SP1 antibody and amplicons covering the distal and proximal region of the *MET* promoter, which contain and lack SP1 sites, respectively (Figure 3.13F). SP1 binding was selectively enriched on the proximal region of *MET* promoter. p53 expression resulted in significant reduction of SP1 binding (Figure 3.13G-H). Conversely, as expected, p53 did not affect SP1 binding to the *survivin* promoter (Figure 3.13G), which is repressed by p53 through recruitment of chromatin remodeling complexes (Esteve et al., 2007). Consistently, in mouse OSE cells, *p53* inactivation led to an increase of SP1 binding to the corresponding mouse *Met* promoter proximal region (Figure 3.13I). Taken together, these results show that p53 is likely to suppress *MET* promoter activity through inhibition of SP1 DNA binding.

3.4.6 Mutant p53 proteins interact with SP1 and their effects on OSE motility and invasion depend on MET.

Figure 3.13 p53 inhibits SP1 binding to *MET* promoter. (A) Effect of mithramycin A (mitA) on *MET* promoter activity. SKOV-3 cells were pretreated with mitA one hr before transfection with 0.27MET promoter construct. (B) Individual and combined effects of mitA (100 nM) and p53 expression on 0.27MET promoter activity in SKOV-3 cells. Bars, SD (n=3). *** $p<0.001$ (C-E) Detection of p53 and SP1 binding by co-immunoprecipitation. Either control (pORF, mock) or p53 expression vector (pORF-hp53, p53) was transfected into SKOV-3 cells. 48 hrs after transfection, cell lysates were immunoprecipitated with SP1 (C) or p53 (D) antibodies followed by Western blotting with p53 or SP1 antibodies, respectively. (E) OVCA433 cell lysates were either immunoprecipitated with SP1 antibodies, followed by Western blotting with p53 antibody (upper, IP:SP1). Samples of the same lysates were used for Western blotting with SP1 or p53 antibody before precipitation (lower, WB). (F) Design of ChIP assays. The proximal region of *MET* promoter contains three potential SP1 binding sites (grey oval shapes), but the distal region has none. Arrows, primers for PCR amplified regions. (G-H). Qualitative (G) and quantitative (H) analyses of ChIP assays with SP1 antibody and the distal and proximal region of *MET* promoter. ChIP assay was performed 48 hrs after transfection of p53 vector into SKOV-3 cells. 2% input control was loaded for comparison. The results are representative of three independent experiments. *P = 0.0182. Bars, SD. (I) Analysis of mouse *Met* promoter region corresponding to the proximal region of human *MET* promoter. ChIP assay was performed 48 hrs after either Ad-*LacZ* or Ad-*Cre* infection of OSE cells from $p53^{fl/fl}$ mice.

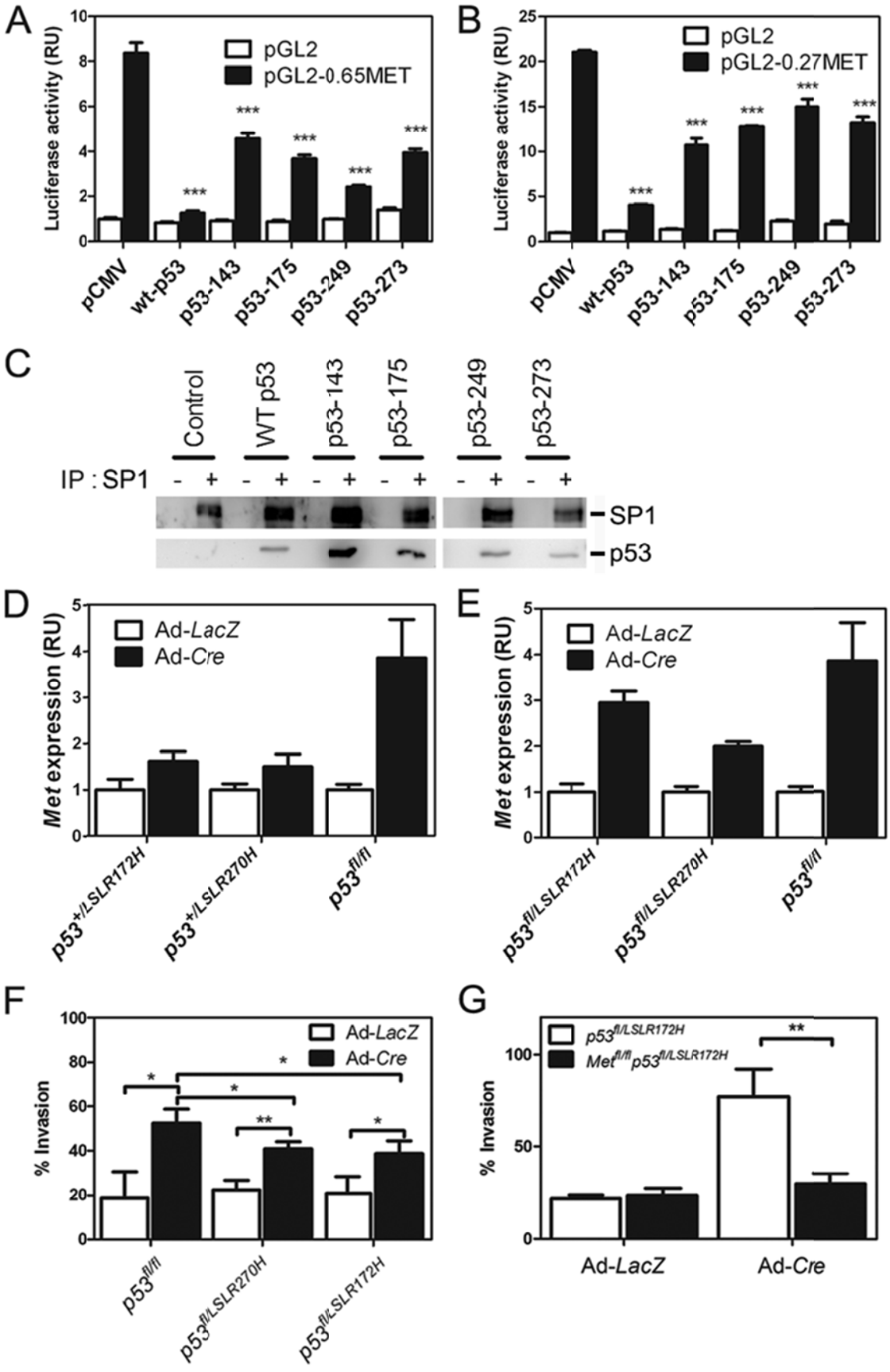

A
Luciferase activity (RU)
pGL2
pGL2-0.27MET
Mithramycin A
100 200 400 1000 nM
B
Luciferase activity (RU)
pGL2-0.27MET
p53
Mithramycin A
***
C
mock p53
IP : SP1
SP1
p53
D
mock p53
IP : p53
SP1
p53
E
IP : SP1
IP : SP1
WB
SP1
p53
OVCA433
F
Distal
Proximal
-2907
100bp
SP1
-369 ~ -360
-304 ~ -296
-197 ~ -188
-42 ~ -33
0
+99 ~ +109
+131
G
mock p53
Input - + Input - + N IP : SP1
Distal
Proximal
survivin
H
mock
p53
SP1 enrichment (%)
No Ab
SP1 Ab
*
I
Ad-LacZ
Ad-Cre
Input
IP : SP1
N
MET
p53fl/fl OSE

To explore the role of mutant p53 in Met regulation, four expression vectors encoding DNA binding domain p53 mutants (V143A, R175H, R249S and R273H) were transfected with *MET* promoter reporter constructs into SKOV-3 and OSN2 cells. All mutant p53 vectors suppressed 0.65MET and 0.27MET constructs, albeit less efficiently compared with wild-type p53 (Figure 3.14A-B and Figure 3.15A-B). Accordingly, the mutant p53 proteins were co-immunoprecipitated with SP1 (Figure 3.14C), indicating that at least these common p53 mutants retain interactions with SP1.

Cells of human ovarian cancer cell lines OVCA432 and OVCAR-3 carrying mutant p53 did not show an increase of already high MET levels after p53 knockdown (Figure 3.15C). To further explore the role of mutant p53 in motility and invasion, primary OSE cells were prepared from $p53^{+/LSLR172H}$ and $p53^{+/LSLR270H}$ mice, which contain a conditionally activated copy of mutant p53 corresponding to human p53 R175H and R273H hot-spot mutations, respectively. Despite overall increased p53 expression after Ad-*Cre* infection (Figure 3.16A), *Met* expression increased only slightly (Figure 3.14D). While loss of both *p53* copies is sufficient to immortalize OSE cells (18), $p53^{+/R172H}$ and $p53^{+/R270H}$ OSE cells had a very limited proliferation potential and underwent senescence (Figure 3.16B), consistent with previously reported phenotype of mouse embryonic fibroblasts (MEFs) carrying the same *p53* mutations (Olive et al., 2004).

Similarly to MEF immortalization after the loss of remaining wild-type copy of *p53*, OSE cells from $p53^{fl/LSLR172H}$ and $p53^{fl/LSLR270H}$ mice were easily immortalized after Ad-*Cre* infection. In these cells levels of Met expression were higher than those in cells heterozygous for mutant p53 but less than those in p53 null background (Figure 3.14E). Cells carrying mutant R172H or R270H p53 also displayed increased cell invasion, although at a lesser extent than that of p53 null cells (Figure 3.14F). Similarly to observations in p53 null cells, deletion of *Met* abrogated increased migration and invasion associated with mutant R172H or R270H p53 (Figure 3.14G and Figure 3.16C).

Figure 3.14 Contribution of mutant p53 protein to OSE motility and invasion depends on MET. (A-B) Effects of wild-type and mutant p53 (V143A, R175H, R249S and R273H) on the activity of 0.65MET (A) and 0.27MET (B) promoter fragments in SKOV-3 cells. (C) Detection of SP1 binding to wild-type and mutant p53 after transient transfection into SKOV-3 cells. (D-E) *Met* expression in OSE cells from $p53^{+/LSLR172H}$, $p53^{+/LSLR270H}$, $p53^{fl/LSLR172H}$ and $p53^{fl/LSLR270H}$ mice 48 hrs after Ad-*Cre* infection. qRT-PCR. n=3, mean ± SD. (F) Invasion of OSE cells from $p53^{fl/fl}$, $p53^{fl/LSLR172H}$ and $p53^{fl/LSLR270H}$ mice 24 hrs after Ad-*Cre* infection. (G) *Met* inactivation abrogates increased invasion of OSE cells containing *p53* R172H mutation. Bars, SD (n=3). *p<0.05; **p<0.01; ***p<0.001.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wt-p53
p53-143
p53-175
p53-249
p53-273
B
Luciferase activity (RU)
pGL2
pGL2-0.27MET
C
Control
WT p53
p53-143
p53-175
p53-249
p53-273
IP : SP1
SP1
p53
D
Met expression (RU)
Ad-LacZ
Ad-Cre
p53+/LSLR172H
p53+/LSLR270H
p53fl/fl
E
Met expression (RU)
Ad-LacZ
Ad-Cre
p53fl/LSLR172H
p53fl/LSLR270H
p53fl/fl
F
% Invasion
Ad-LacZ
Ad-Cre
p53fl/fl
p53fl/LSLR270H
p53fl/LSLR172H
G
% Invasion
p53fl/LSLR172H
Metfl/flp53fl/LSLR172H
Ad-LacZ
Ad-Cre

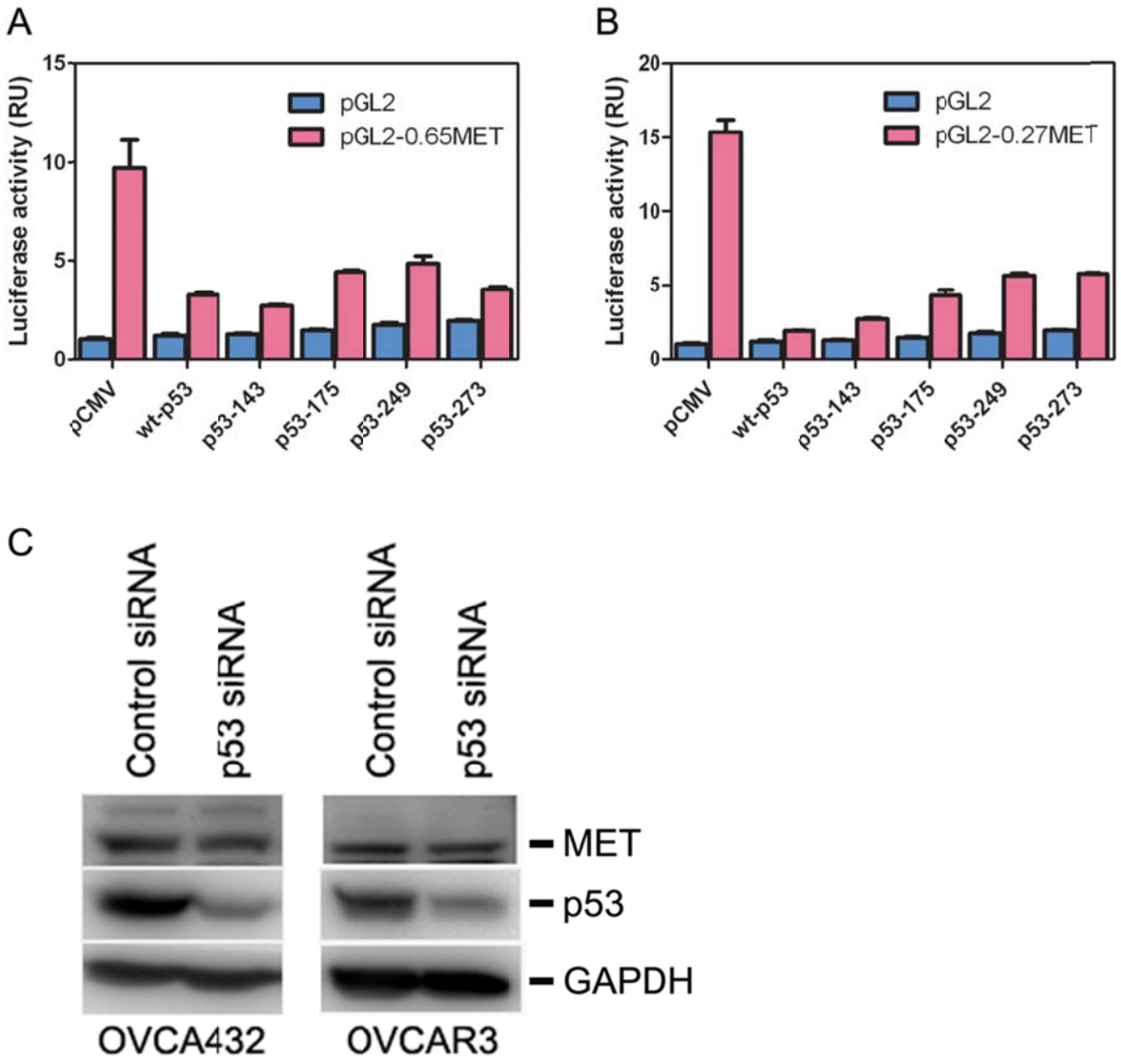
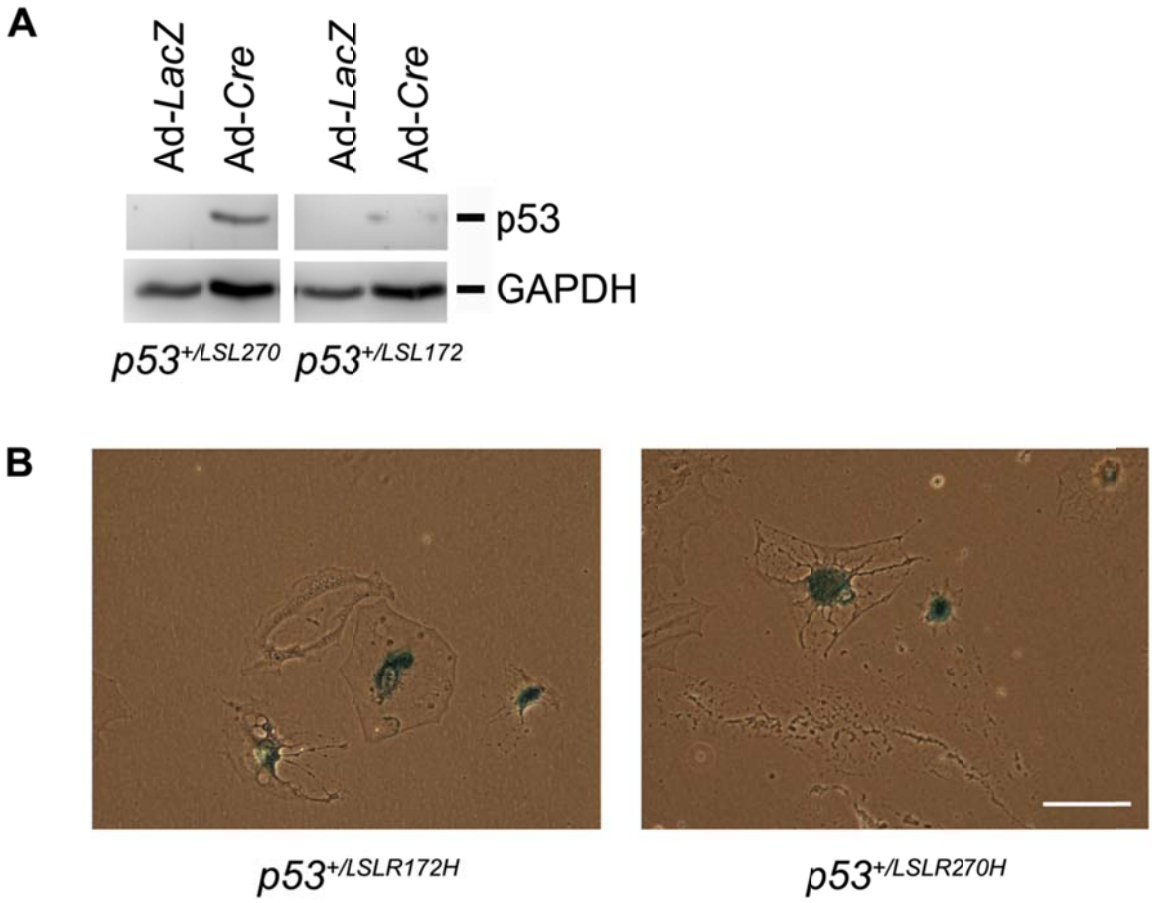


Figure 3.15 The effect of mutant p53 on *MET* expression. (A and B) 0.65MET (A) and 0.27MET (B) promoter constructs were co-transfected with wild-type or mutant p53 into OSN2 cells. Bars, SD (n=3). (C) 20 nM of either control or p53 siRNA was transfected into OVCA432 and OVCAR-3 cells and MET expression was measured by Western blotting 48 hrs afterward.

Figure 3.16 The role of mutant p53 in OSE cells. (A) OSE cells from *p53*$^{+/LSLR172H}$ or *p53*$^{+/LSLR270H}$ mice were infected by either Ad-*LacZ* or Ad-*Cre*. Slight increase of p53 was detected, likely because of mutant p53 expression in Western blot analysis. (B) OSE cells expressing mutant p53 were not immortalized and underwent senescence. Staining for senescence-associated β-galactosidase. Scale Bar, 500 μm. (C) Either Ad-*LacZ* or Ad-*Cre* infected OSE cells from *p53*$^{fl/LSLR270H}$ and *p53*$^{fl/LSLR270H}$*Met*$^{fl/fl}$ mice were seeded on a control insert or Matrigel insert with serum free media and exposed to complete growth media containing 20 ng/ml HGF in the lower chamber. Migrating and invading cells were counted 20 hrs after staining under the microscope. Bar, SD, n=3. **p<0.01. N.S. indicates 'not significant'.


A
Ad-LacZ
Ad-Cre
Ad-LacZ
Ad-Cre
p53
GAPDH
p53+/LSL270
p53+/LSL172
B
p53+/LSLR172H
p53+/LSLR270H
C
p53fl/LSLR270H
Metfl/flp53fl/LSLR270H
% Invasion
150
100
50
0
N.S.
**
Ad-LacZ
Ad-Cre

3.5 Discussion

Our work shows that p53 controls the expression of the proto-oncogene *MET* by two mechanisms consisting of suppression of MET on the transcriptional level via promoter repression and on the post-transcriptional level via transactivation of miR-34. Contrary to a previous report (Seol et al., 1999), we were unable to find any canonical or novel p53 binding sites selectively responsible for either activation or repression of *MET* promoter. At the same time, the results of our promoter analysis, together with co-immunoprecipitation and ChIP assays, provide support for a mechanism of MET transcriptional repression based on inhibition of SP1 binding to DNA through physical interactions between p53 and SP1 rather than on direct promoter binding by p53. Consistent with this possibility, it has been previously reported that SP1 activates *MET* promoter activity (Verras et al., 2007; Zhang et al., 2003) and interacts with wild-type (Koutsodontis et al., 2001; Wierstra, 2008) and mutant p53 (Brosh and Rotter, 2009). Furthermore, p53 inhibits SP1 DNA binding on *HIV-LTR* and *MGMT* promoter in vitro (Bargonetti et al., 1997; Bocangel et al., 2009).

Either lack of p53 or expression of its mutant forms abrogate *miR-34* transactivation (Hermeking, 2007). Therefore, both types of mutations result in elevation of miR-34-dependent MET expression. However, our study shows that unlike null mutations, mutant p53 protein retains *MET* promoter suppressive function, albeit to a lesser degree than wild-type p53. Consistent with the lesser extent of MET expression, cells expressing mutant p53 display a lower MET-dependent motility and invasion as compared to null mutants. These findings suggest that specific alterations in individual components of p53/MET signaling network may modulate the course of pathological process. Consistent with this possibility, the poorest

prognosis of *p53* null mutation has been reported in some types of neoplasms, including ovarian, lung and breast cancers (Hashimoto et al., 1999; Lai et al., 2004; Sood et al., 1999).

Recent studies indicate that lack of p53 versus expression of mutant p53 proteins may have a different impact on motility and invasion in the context of particular cell lineages as well as additional genomic alterations. Our observations of increased motility and invasion by p53 null OSE cells are consistent with previous studies that have shown comparable effects in p53 null immortalized fibroblasts and MEFs (Alexandrova et al., 2000; Gadea et al., 2007). Similarly, non-small cell lung carcinoma line NCI-H1299 cells harboring a *p53* null mutation have been reported to migrate faster than those expressing R175H mutant p53 (Kalo et al., 2007).

At the same time, some investigators have reported that mutant p53 protein but not null *p53* mutations may drive cell motility and invasion by promoting integrin recycling (Muller et al., 2009), by forming a complex with Smad to oppose p63-mediated control of putative metastasis suppressors Sharp-1 and cyclin G2 (Adorno et al., 2009) or by stabilizing the invasion promoter Slug (Wang et al., 2009). Consistent with cell-type specificity of p53 effects, mice expressing mutant p53 develop a more diverse spectrum of neoplasms as compared to null mutants (Olive et al., 2004). Interestingly, in line with observations by Grugan et al. (Grugan et al., 2010), knockdown of mutant p53 in ovarian carcinoma cells did not change levels of MET expression. This observation is at variance with our results on primary OSE cells carrying conditional mutant *p53* allele, which may indicate that established cancer lines and neoplastic cells at advanced cancer stages develop additional mechanisms ensuring MET signaling in cells carrying mutant forms of p53 protein. Assessment of primary ovarian cancer cells should address this problem in future studies. It should be also of interest to evaluate p53/MET signaling in the fallopian tube epithelium, another potential cell of origin of serous adenocarcinoma of the ovary (Kindelberger et al., 2007; Piek et al., 2001).

There are indications that some additional genetic or epigenetic alterations are required for stabilization and accumulation of mutant p53 thereby leading to gain-of-function phenotype (Terzian et al., 2008). In agreement with this possibility, our results in primary OSE from *p53*$^{+/R172H}$ or *p53*$^{+/R270H}$ mice, as well as studies by others in MEFs with the same genotypes (Olive et al., 2004), show that cell immortalization is acquired only after the loss of the remaining wild-type copy of *p53*. Consistently, Adorno et al. (Adorno et al., 2009) were able to observe increased migration of cells expressing mutant p53 only in combination with HRAS and TGFβ signaling. Notably RAS mutations are extremely rare in high grade serous ovarian carcinoma (Bast et al., 2009).

Development of therapies aimed at correction of the p53 pathway remains among the most coveted goals in cancer research (Brown et al., 2009). Importantly, our experiments have demonstrated that MET is a critical component of motility and invasion in cells either lacking p53 or expressing its mutant forms. Therefore, treatment of cancers with p53 mutations is likely to benefit from therapeutics aimed at MET, such as small targeting molecules (Comoglio et al., 2008). This finding is of particular significance because, despite successful outcomes in cell culture and animal in vivo experiments, direct re-introduction of the p53 gene failed in clinical trials, including EOC (Zeimet and Marth, 2003). Our study also indicates that approaches aimed at indirect elimination of mutant p53 protein (e.g., by p53 siRNA) should be avoided in cancers with active p53/MET signaling network. The in-depth understanding of mechanisms by which p53 regulates MET in the context of different cell types, as well as specific p53 mutations, may be essential for future development of individualized therapeutics.

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CHAPTER 4

GENERATION AND CHARACTERIZATION OF MICE WITH CONVENTIONAL AND CONDITIONAL INACTIVATION OF MIR-34 FAMILY

4.1 Abstract

Genes encoding for miR-34 family have been shown to be a direct target of transcriptional activation by p53 tumor suppressor. Since miR-34 is highly evolutionarily conserved and targets multiple proteins involved in cell cycle (Cdk4, Cdk6, Cyclin D1, E2F3), stem cell maintenance (Notch1 and 2, EZH2), metabolism (SIRT1), cell motility (Met), apoptosis (Bcl-2) and induced pluripotent stem (iPS) cell reprograming (c-Myc and Klf4), it has been expected to play a variety of essential regulatory roles in a number of important biological processes. Furthermore, since miR-34 is deleted or downregulated in many cancers, it has been proposed to have a tumor suppressor function. However, direct genetic testing of specific roles of miR-34 family during development and carcinogenesis has not been performed so far. Furthermore, it remains unclear to which extent miR-34 contributes to p53 effects, such as DNA damage response and iPS cell reprogramming. To address these issues we have prepared mice carrying conventional and conditional alleles of *mir-34a* and *mir-34b/c*. We report that similarly to p53 deficient phenotype, mice lacking all *mir-34*genes are viable. However, at variance to p53 mice, *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ *mice* do not develop lymphomas within first 3 months of age but exhibit thymic and splenic hypoplasia, ulceration of forestomach and focal hyperplasia of Purkinje neurons. Furthermore, in spite of accelerated proliferation, which is similar to that of p53 deficient cells,

mir-34 deficient cells do not show increased reprogramming efficiency, indicating that this parameter is insufficient for facilitation of reprogramming by p53. *mir-34b/c* deficiency also caused radioresistance to 15 Gy ionizing radiation (gastrointestinal tract syndrome) but not 10 Gy (hematopoietic syndrome), contrary to known radiosensitivity and radioresistance, respectively, of p53 deficient mice. Taken together, these findings indicate that in spite of direct regulation by p53, miR-34 has a significant set of distinct biological functions. Availability of mice with conventional and conditional inactivation of all miR-34 family members will provide the necessary basis for further detailed characterization of *mir-34* biological functions and determining their place within p53 signaling network during development and carcinogenesis.

4.2 Introduction

microRNAs (miRNAs) are a class of small non-coding RNAs that function to control gene expression. Recent studies have shown the tumor suppressive and oncogenic potential of a number of miRNAs, underscoring their importance in human cancer therapeutic and diagnostic applications (Reviewed in (Corney and Nikitin, 2008; Farazi et al., 2011; Reddy et al., 2010)). One of the most intensively studied miRNAs is miR-34 family, which is among the most evolutionary conserved miRNAs (Grad et al., 2003). miR-34 family is encoded by the *mir-34a* gene located on human chromosome 1 and *miR-34b* and *mir-34c* genes located on human chromosome 11 (chromosomes 4 and 9, respectively, in mouse). miR-34a is transcribed alone, while miR-34b and miR-34c share a common primary transcript as a cluster (Corney and Nikitin, 2008). The first evidence for potential involvement of miR-34 family in cancer has arisen from reports of a minimal deleted region 11q23-q24-D containing *mir-34b/c* in lung and breast cancers (Calin et al., 2004), and 1p37 containing *mir-34a*, frequently deleted in high-risk

neuroblastoma (Cole et al., 2008; Welch et al., 2007). In 2007 several groups, including our laboratory, independently reported that miR-34 family is a direct target of p53 transactivation (Bommer et al., 2007; Chang et al., 2007; Corney et al., 2007; He et al., 2007; Tarasov et al., 2007). Loss or decreased expression of miR-34 has been reported in multiple cancer cell lines and human malignancies (summarized in Table 1.2). The high frequency of miR-34 downregulation in human cancer and its correlation with advanced stages of the diseases and poor prognosis suggests that genes of *mir-34* family are putative tumor suppressors. In addition, the tumor suppressive role of miR-34a has been shown by *in vivo* delivery in lung and pancreatic cancer models (Pramanik et al., 2011; Trang et al., 2011). However, a direct genetic proof of *mir-34* family's role as tumor susceptibility/suppressor genes has been missing.

Ectopic expression of miR-34 has been shown to regulate cell cycle, apoptosis, senescence, cell migration and invasion through regulating target genes (Reviewed in (Hermeking, 2010)). Validated targets for cell cycle regulation include CDK4, CDK6, Cyclin E2 and Cyclin D1, associated with G1-arrest (Bommer et al., 2007; Fujita et al., 2008; He et al., 2007; Sun et al., 2008). As an important target for apoptosis and DNA damage response, Bcl-2 is a validated target of entire miR-34 family members (Ji et al., 2009). As described in Chapter 2 and 3, anti-migratory and anti-invasive effect of miR-34 appears to be mainly through posttranslational regulation of MET proto-oncogene (Corney et al., 2010; He et al., 2007; Migliore et al., 2008). In addition, miR-34 family is also involved in a various cellular responses such as senescence (Sirt1) and stem cell maintenance (Notch1, 2 and Jagged-1) (Li et al., 2009b; Pang et al., 2010; Yamakuchi et al., 2008). Particularly, the involvement of miR-34 family control of stemness genes suggests that miR-34 family may play a role in regulation of normal and neoplastic stem cells as well as iPS cell reprogramming process.

An important finding to understand the mechanism of iPS cell reprogramming process was the involvement of p53 (Menendez et al., 2010). Consistently, miR-34a is also known to

target c-Myc (Cannell et al., 2010; Christoffersen et al., 2010), one of the initially identified reprogramming factors (Takahashi and Yamanaka, 2006). Interestingly, miR-34a inhibition in embryonic stem cells has been shown to delay cell differentiation in LIF free condition (Tarantino et al., 2010), suggesting the potential role of miR-34a in stem cell self-renewal and differentiation. Thus, it is reasonable to hypothesize that the p53-mediated barrier to reprogramming may be due to miR-34 family at least in part. However, it remains unknown if inactivation of *mir-34* may promote the reprogramming of somatic cells into iPS cells.

p53 activation followed by initiation of cell apoptosis or senescence in response to DNA damage is one of the key tumor suppression mechanisms, because DNA damage and genomic instability commonly accompany tumor progression (Meek, 2009). Since genotoxic insults induce expression of miR-34 family in p53-dependent manner it is possible that miR-34 is involved in mediating p53 downstream signaling leading to cell apoptosis or senescence in a cell type specific manner. Consistent with a cell type specificity of DNA damage response, in *C. elegans mir-34* mutation caused radiosensitive phenotype in the soma and radioresistance in the germline (Kato et al., 2009). At the same time, ectopic expression of miR-34a resulted in radiosensitivity in colon cancer cell line (Liu et al., 2011) but radioresistance in breast cancer cell line (Kato et al., 2009). The precise role of miR-34 in DNA damage response in mammals *in vivo* remains to be determined.

In order to study the precise role of miR-34 family in physiologically relevant settings, we decided to inactivate *mir-34* genes in the mouse. Here we report the generation of mice with conventional and conditional deletion of *mir-34a*, as well as preparation and initial characterization of mice deficient for entire *mir-34* family. We demonstrate that mice lacking all *mir-34* genes are alive but show defects in several systems, such as hypoplasia of the thymus and the spleen, ulceration of the forestomach and focal hyperplasia of Purkinje neurons. Loss of *mir-34* resulted in increased cell proliferation but did not improve iPS cell reprogramming

efficiency. In addition, *mir-34b/c* deficiency caused radioresistant phenotype upon 15 Gy IR, suggesting that miR-34 is involved in DNA damage response. Taken together, these findings indicate that miR-34 family has a number of biological functions which do not recapitulate those of p53.

4.3 Materials and methods

BAC recombineering and construction of mir-34a targeting vector. The conditional gene-targeting vector was constructed using a recombineering approach as described elsewhere (Liu et al., 2003). Detailed recombineering protocols, plasmids (PL253, PL451 and PL452) and *E.coli* strains (EL250 and EL350) can be found on website at http://recombineering.ncifcrf.gov. Mini-targeting vector 1 which contains two homology arms flanking 12 Kb genomic sequence of *pre-mir-34a* to be subcloned by gap repair was cloned into PL253 plasmid. The primers used for amplification of the 5' and 3' homology arms were as follows: 5' arm F, 5' AGTCG CGGCC GCGCT AAATG AGGAC TTCGT GACAA A 3'; 5' arm R, 5' CAATC AGTTC ACAAC CACTT GGTA 3'; 3' arm F, 5' AGGGA TCCCA CAGGC AAGGG TGTGG GTTCA 3'; and 3' arm R, 5' ATCTA GATTT CTCAA AATGT CACCA GTGTT CA 3'. Mini-targeting vector 2 which contains two homology arms for 5' of *pre-mir-34a* was cloned into PL452 plasmid. The primers used for amplification of the 5' and 3' homology arms were as follows: 5' arm F 5' AGGGT ACCAG CCAGC AGGGA ATAGT GGTCA 3'; 5' arm R, 5' AGAAT TCCAC ATCAG GCAAA ACACG TATAC A3'; 3' arm F, 5' AGGGA TCCTA TACAC TGTGT GTGTG CCTGG TA 3'; and 3' arm R, 5' AGTCG CGGCC GCCCC AGGCT GAGGC GCCCG CA 3'. Mini-targeting vector 3 which contains two homology arms for 3' of *pre-mir-34a* was cloned into PL451 plasmid. The primers used for amplification of the 5' and 3' homology arms were as follows: 5' arm F 5' AGGGT

ACCAT CTTTA CCCAC TGAAC CATCC CGA 3'; 5' arm R, 5' AGAAT TCCTC GTGTG TGCGG CTTCG GGCA 3'; 3' arm F, 5' AGGGA TCCGC TTCAC ATTTT TTCAG TTTTC CTA 3'; and 3' arm F, 5' AGTCG CGGCC GCGCC TTGTA CTTGG AGAGA TCCA 3'.

Embryonic stem (ES) cell culture. The E14 mouse embryonic stem cell line was cultured in undifferentiating conditions on mitotically inactivated feeder cells and in the presence of leukemia inhibitory factor (LIF) at 37°C in 10% CO_2. Briefly, medium used was Dulbecco's Modified Eagle Medium (high glucose) containing 15% heat-inactivated ES cell qualified fetal bovine serum, 6 mM L-glutamine, 1 mM sodium pyruvate, 1x Dulbecco's non-essential amino acids (all purchased from Invitrogen, Carlsbad, CA) plus 10^{-4} M 2-mercaptoethanol (Sigma, St. Louis, MO), and 10^3 units/ml LIF (Millipore, Billerica, MA). For positive and negative selection, cells were cultured in the medium containing 200 μg/ml G418 and 0.2 μM Ganciclovir.

Primary cell cluture. Mouse embryonic fibroblast (MEF) cells were prepared from 13.5-15.5-day old C57Bl/6 embryos carrying neomycin cassette under control of PGK promoter (C57BL/6J-Tg(pPGKneobpA)3Ems/J, The Jackson Laboratory, Bar Harbor, ME; stock number 002356) and prior to use as feeder cells mitotically inactivated by exposure to 10 μg/ml mitomycin C for one hour at 37°C followed by three washes with phosphate-buffered saline (PBS). ES cells were routinely passaged using 0.25% trypsin/EDTA prior to reaching confluence. Feeder cells were separated from ES cells by differential attachment to gelatinized plates for one hour at 37°C. For tail-tip fibroblasts (TTFs) culture, 1 cm length of tail-tip was cut, minced and incubated with DMEM containing 10% FBS, 6 mM L-glutamine, 1 mM sodium pyruvate and penicillin/streptomycin. Lipofectamin 2000 reagent (Invitrogen, Carlsbad, CA) was used for the transfection following manufacturer's recommendations.

Mice. mir-34b/c conditional and conventional knock-out mice were generated by Dr. David Corney in our laboratory (Corney, 2010). While *mir-34a* conditional and conventional knock-out mice were being generated in by us, *mir-34a* conditional knock-out mice were independently generated and provided to us by our collaborator Dr. Heiko Hermeking (Ludwig-Maximilians-University Munich). Hermeking's mice were used for OSE experiments (described in Chapter 3), pathological evaluation and iPS cell reprogramming in this study. Transgenic mice with carrying *Flp* transgene (129S4/SvJaeSor-*Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$/J) and *Cre* transgene (FVB/N-Tg(EIIa-cre)C5379Lmgd/J) were purchased from Jackson laboratory (Bar Harbor, Maine). Deletion of the Neo cassette was obtained by crossing to *Flp* transgenic mice. Both ours and Hermeking's *mir-34a*$^{loxP/loxP}$ mice were crossed with *mir-34b/c*$^{loxP/loxP}$ mice to obtain mice with knockout of all *mir-34* genes. Additionally they were crossed with *Cre* transgenic mice (EIIa-Cre) to obtain mice with conventional *mir-34* knockout. All of the mice were maintained identically, following recommendations of the Institutional Laboratory Animal Use and Care Committee.

Body weight measurements. Body weights of *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ female mice (n=5; 13, 24, 32, 42 and 73 days old) and similar ages of *mir-34a*$^{loxP/loxP}$ *mir-34b/c* $^{loxP/loxP}$ female mice (n=7) were measured under identical conditions at the same time of the day.

Pathological Analyses. Mice were subjected to careful evaluation during necropsy and the brain, lung, thymus, stomach, small intestine (the proximal and distal part), large intestine (colon), kidney, ovary, uterus, spleen, pancreas, mammary gland, heart and thigh muscle were collected for histological processing. Tissues were placed into PBS-buffered 4% paraformaldehyde, fixed overnight at 4ºC and embedded in paraffin. Prepared 4 µm paraffin sections were stained with

Hematoxylin and Eosin and subjected to pathological evaluation. Sections of the proximal region (6 cm distally to the stomach) and the distal region (2 cm proximal to the cecum) of the small intestine were prepared as described by (Kirsch et al., 2010). All tissues and organs were collected from *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ female mice and age- and sex-matched wild-type female mice. Since age- and sex-matched wild-type littermates for *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ mice from *mir-34a*$^{+/-}$ *mir-34b/c*$^{+/-}$ crosses are expected to have low frequency, age- and sex-matched *mir-34a*$^{loxP/loxP}$ *or mir-34b/c*$^{loxP/loxP}$ mice were also used as control.

PCR for genotyping. 5' arm of *mir-34a* containing a *loxP* site was amplified by using F 5' GAATG TGTAT ACGTG TTTTG CCTGA 3' and R 5' TAAC TCCAG TTACA GGGAC TCTGA 3'. Wild-type allele generated 100 bp, and targeted allele 198 bp, shown in Figure 4.3.C. In order to amplify wild-type, floxed allele and null allele together, multiplex PCR was done by using three primers (1) F 5' GAATG TGTAT ACGTG TTTTG CCTGA 3', (2) F 5' AGCTG ACATG CCAGG AATGC TGA 3', and (3) R 5' TGGCC CCTTT AATTT ACAAG CCCA 3'. Wild-type allele generates 196 bp, floxed 293 bp, and null 216 bp respectively, shown in Figure 4.4.

Southern blotting. Genomic DNA was isolated from ES clones in DNA lysis buffer (100mM NaCl, 50mM Tris-HCl pH 7.5, 10mM EDTA pH 8.0, 0.5% SDS and 100 µg/ml Proteinase K). Genomic DNA was digested by SphI, resolved in 0.8% Agarose gel, and transferred to Zeta probe membrane (BioRad, Hercules, CA). Genomic DNA sequence for probe region was PCR-amplified by using primers F 5' GTGAC ATTTT GAGAA ATTGA AACAA 3' and R 5' ACCTT ATAAA AGAAG ACAGC ACCA 3'. PCR product was cloned into T-vector, digested by restriction enzyme, and gel-purified. Southern blotting probe was prepared by ^{32}P-dCTP end-labeling. Purified probe was hybridized with membrane in Church buffer (7% SDS, 1% bovine

serum albumin, 1mM EDTA, Na_2HPO_4 0.25M, pH 7.2). After washing, membrane was exposed to phosphorscreen.

Ionizing irradiation (IR). Mice were placed in irradiation chambers and whole-body radiation was performed with a sealed ^{137}Cs source irradiator Mark 1-68 (JL Shepherd and Associates, San Fernando, CA) at a dose rate of approximately 120.3 cGy/min. Irradiated mice were monitored twice a day and euthanized as scheduled or at the time of becoming moribund.

In situ hybridization. Detection of miR-34a was done by the protocol adapted from Nelson et al. (Nelson et al., 2006). To prevent the loss of miRNAs, we additionally applied 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) fixation as described by Pena et al. (Pena et al., 2009). In brief, 4-μm-thick sections of formalin fixed, paraffin-embedded material were deparaffinized, rehydrated, and fixed with EDC. After 1-h prehybridization, a digoxigenin (DIG)–labeled locked nucleic acid (LNA) probe (Exiqon, Woburn, MA) was hybridized to proteinase K–treated sections at 56°C for 16 h. Slides were then incubated with anti-DIG-AP antibody (Roche, Indianapolis, IN), and miRNA expression was detected by nitrobluetetrazolium/5-bromo-4-chloro-3-indolyl phosphate method. Methyl green was used for nucleic counterstaining.

iPS cell reprogramming. Reverse tetracycline transactivator (R26-M2rtTA) construct driven by the ROSA26 promoter and doxycycline-inducible Oct-4, Klf-4, Sox-2 and c-Myc lentiviral constructs were described by (Brambrink et al., 2008). In brief, 293T cells were transfected with a mixture of viral plasmid and packaging constructs expressing the viral packaging functions and the VSV-G protein. Medium was replaced 24 hours after transfection, and viral

supernatants were collected at 48 hours. TTFs or MEFs were incubated with viral supernatants and fresh media at a ratio of 1:1 for 24 hours according to commonly used methods at Cornell Induced Pluripotent Stem Cell Core. 6.4 x 10^4 TTFs and 7.2 x 10^4 MEFs were plated on 6 cm tissue culture dish and doxycycline (1.5 μg/ml final concentration) was daily added to the cell culture medium starting from 1 day following the split. Cell number for growth analysis was measured 1 and 2 days after doxycycline treatment.

Alkaline phosphatase (AP) and Nanog staining. AP staining kit (Vector Laboratories, Burlingame, CA) was used according to manufacturer's instructions. For Nanog staining, standard immunohistochemistry protocol was applied as described elsewhere (Nikitin and Lee, 1996). Briefly, rabbit polyclonal antibody against Nanog (1:50, #80892, 1 hour incubation at room temperature, Abcam, Cambridge, MA) was detected by goat anti-rabbit biotinylated secondary antibody and followed by ABC-Elite (Vector laboratories, Burlingame, CA). The efficiency of reprogramming was calculated as the relative change compared to that of control experiments.

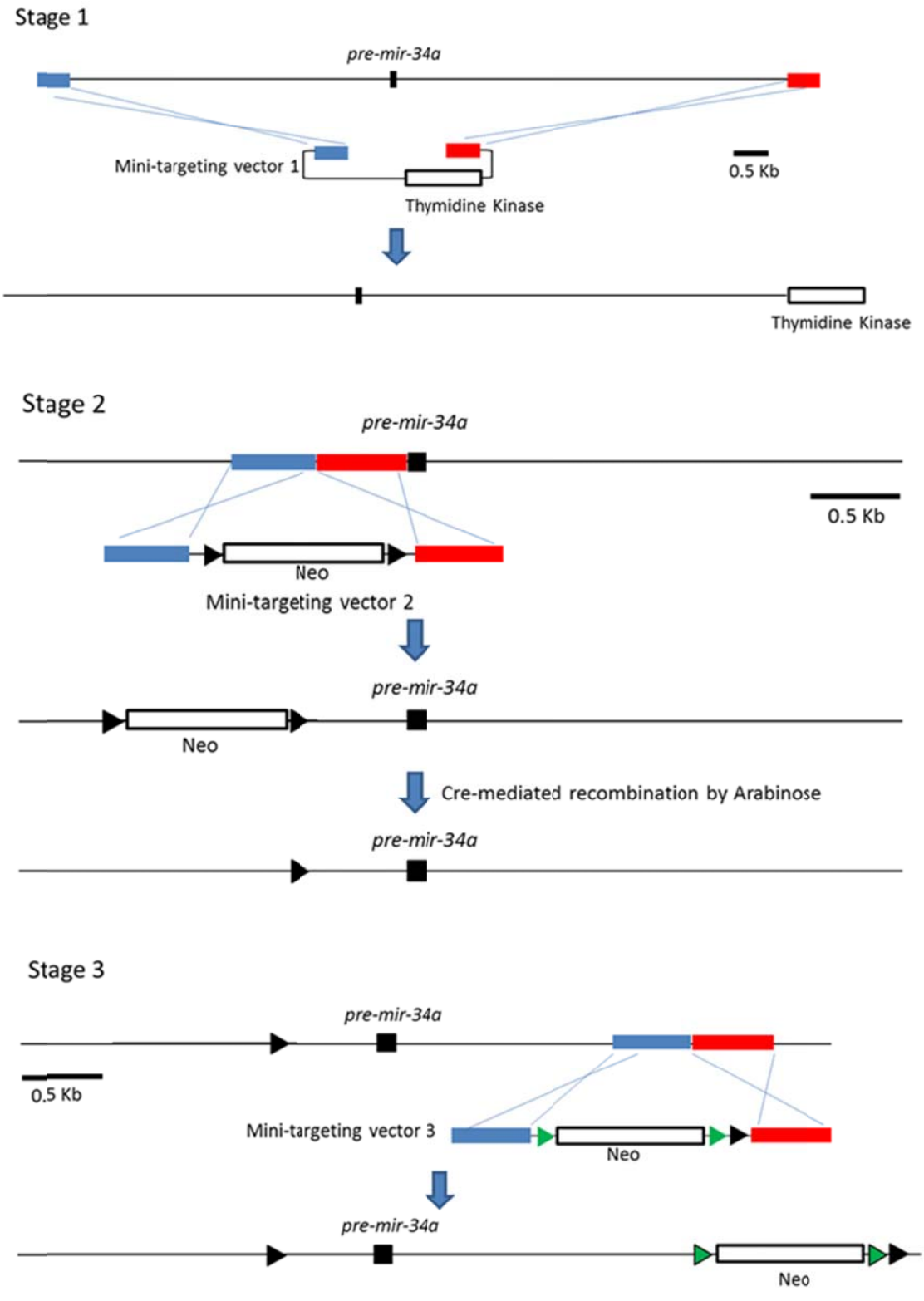
Statistics. Statistical and survival analyses were performed with InStat 3.10 and Prism 5.01 software (GraphPad, Inc., San Diego, CA).

4.4 Results

4.4.1 Preparation of mir-34a$^{loxP/loxP}$ targeting construct by BAC recombineering

In order to generate *mir-34a* targeting construct, BAC recombineering technology was used. RP23-410P10 BAC clone (C57Bl/6 genomic DNA; BACPAC Resource Center at Children's Hospital Oakland Resarch Institute, California) containing *mir-34a* locus was electroporated into EL350 *E.coli* cells. BAC integrity was confirmed by PCR and restriction enzyme digestion pattern analysis. The procedure of *mir-34a* targeting vector construction consists of 3 steps: (1) the retrieval of genomic DNA sequence containing *mir-34a* locus with Thymidine kinase cassette, (2) insertion of one *loxP* site in 5' of *mir-34a* locus, and (3) insertion of *FRT*-flanked Neo cassette with one *loxP* site (Figure 4.1). In order to obtain 12 Kb genomic sequence containing *mir-34a* locus and insert Thymidine kinase cassette for negative selection as a first step, mini-targeting vector 1 which has a PL253 backbone and 500 bp 5' and 3' homologous arms was generated. Linearized mini-targeting vector 1 was electroporated into heat shocked EL350 *E.coli* containing a RP23-410P10 BAC sequence. Ampicillin-resistant clones were selected, and *mir-34a*-TK plasmid was obtained. In order to insert a *loxP* site in 5' arm of *mir-34a* locus, mini-targeting vector 2 which contains PL452 backbone and 500 bp 5' and 3' homologous arms was generated. Linearized mini-targeting vector 2 was PCR-amplified, digested by restriction enzyme, gel-purified and co-electroporated with linearized *mir-34a*-TK plasmid into heat shocked EL350 *E.coli*. Kanamycin-resistant clones were selected, and *loxP*-Neo-*loxP*-*mir-34a*-TK plasmid was obtained. Electroporation of *loxP*-Neo-*loxP*-*mir-34a*-TK plasmid into heat shocked and arabinose treated EL350 *E.coli* cells resulted in Cre recombinase expression and Neo cassette removal. As a result, *loxP*-*mir34a*-TK plasmid was obtained. In order to insert a *FRT*-flanked Neo cassette with a *loxP* site in 3' of *mir-34a* locus, mini-targeting vector 3 which has a PL451 backbone and 500 bp 5' and 3' homologous arms was generated. Linearized mini-targeting vector 3 was PCR-amplified, digested by restriction enzyme, gel-purified and co-electroporated with linearized *loxP*-*mir-34a*-TK plasmid. Kanamycin-resistant clones were selected, and final *mir-34a* targeting construct was obtained. To validate *loxP*-Cre and *FRT*-Flpe recombination, *mir-34a* targeting construct was electroporated into heat-shocked

Figure 4.1 *mir-34a* targeting strategy. The *mir-34a* allele was conditionally targeted by homologous recombination to introduce *loxP* sites flanking the *pre-mir-34a* sequence. In the first step, around 12Kb of genomic DNA sequence containing *pre-mir-34a* was gap-retrieved into PL253 backbone plasmid by homologous recombination. In the second step, a neomycin cassette flanked by *loxP* sites (black triangle) was inserted, and removed by arabinose-inducible Cre recombinase expression, which allows one *loxP* site in 5' of *pre-mir-34a*. In the third step, a neomycin cassette flanked by *FRT* sites (green triangle) with a *loxP* site was inserted in 3' of *pre-mir-34a*.


Stage 1
pre-mir-34a
Mini-targeting vector 1
0.5 Kb
Thymidine Kinase
Thymidine Kinase
Stage 2
pre-mir-34a
0.5 Kb
Neo
Mini-targeting vector 2
pre-mir-34a
Neo
Cre-mediated recombination by Arabinose
pre-mir-34a
Stage 3
pre-mir-34a
0.5 Kb
Mini-targeting vector 3
Neo
pre-mir-34a
Neo

and arabinose-treated EL350 and EL250 respectively, and correct recombination was confirmed by restriction enzyme digestion pattern analysis (Figure 4.2).

4.4.2 Gene targeting and generation of mice containing conditional mir-34a allele

Linearized *mir-34a* targeting vector was electroporated into E14 mouse ES cells (Figure 4.3A), and cultured on the top of feeder cells with G418 (200 μg/ml) and Ganciclovir (0.2 μM) for positive and negative selection respectively. ES clones (n=293) were picked up, and screened for correct homologous recombination by Southern blotting (Figure 4.3B). Clone #241 underwent correct homologous recombination, and further confirmed by PCR analysis (Figure 4.3C). Correctly targeted clone (#241) harboring both the single 5' *loxP* site and the 3' *Frt-loxP*-flanked neo cassette were injected into C57BL/6 blastocysts, using standard procedures. After germ line transmission of the targeted allele, the null and conditional alleles were obtained by crossing to EIIa-*Cre* and *Flp* transgenic mice, respectively (Figure 4.4).

4.4.3 Characterization of mir-34a$^{-/-}$ mir-34b/c$^{-/-}$ mice during development

mir-34a$^{-/-}$ mir-34b/c$^{-/-}$ mice were obtained from crosses between *mir-34a$^{-/-}$* and *mir-34b/c$^{-/-}$* mice. *mir-34a$^{-/-}$ mir-34b/c$^{-/-}$* mice were born alive and phenotypically indistinguishable from *mir-34a$^{loxP/loxP}$ mir-34b/c$^{loxP/loxP}$* mice. *mir-34a$^{-/-}$ mir-34b/c$^{-/-}$* mice were born at the expected Mendelian ratio (Table 4.1, 4.2 and 4.3), and had similar body weights (Figure 4.5) and no gross abnormalities. To confirm loss of miR-34 expression, brain tissues and ovarian surface epitheli-

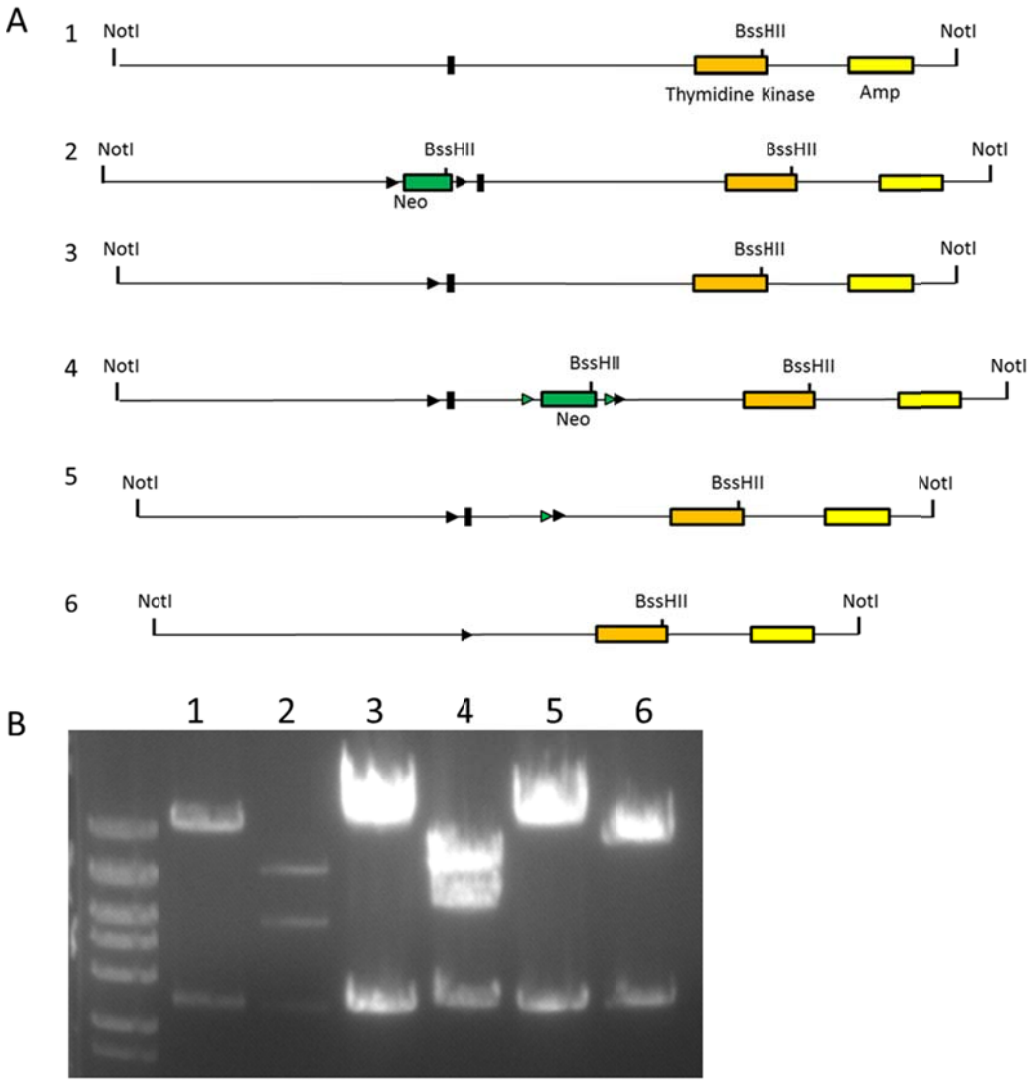
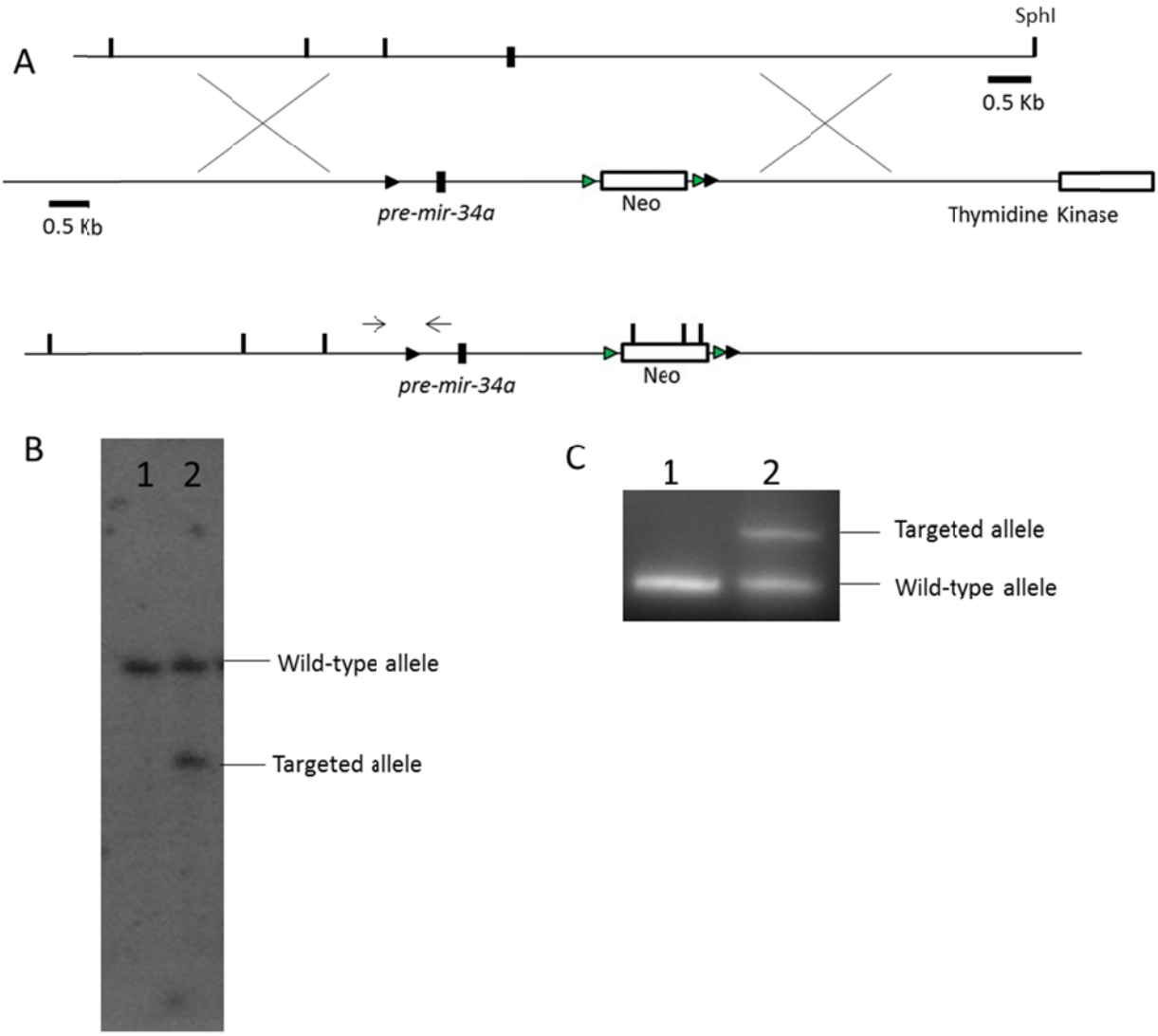


Figure 4.2 Validation of *mir-34a* conditional targeting construct in *E.coli*. A. Each step of *mir-34a* targeting construct was drawn and restriction enzyme recognition sites for NotI and BssHII was indicated. Black and green arrows indicate *loxP* sites and *FRT* sites respectively. Green, orange and yellow boxes indicate neomycin, thymidine kinase and ampicillin resistance gene cassettes respectively. B. Each step of *mir-34a* targeting vector plasmid was digested with NotI and BssHII, and resolved in 0.8% agarose.

Figure 4.3 *mir-34a* targeting strategy and confirmation of correct homologous recombination. A. Schematic diagram of targeting of conditional *mir-34a* allele. Black and green arrowheads indicate *loxP* and *FRT* sites, and SphI enzyme recognition sites are indicated. Black arrows indicate genotyping PCR region. B. Southern blotting of 3' arm (external probe). Genomic DNA from ES clones was isolated, digested with SphI, resolved with 0.8% agarose, transferred onto the membrane, and hybridized with radio-labeled probe. 9.4 Kb band indicates wild-type allele, and 5.2 Kb band a targeted allele. C. PCR genotyping confirms the presence of a *loxP* site in 5' of *pre-mir-34a*. 100 bp band indicates wild-type allele, and 198 bp a targeted allele (lane 1: ES clone without correct homologous recombination, lane 2: ES clone #241 that underwent correct homologous recombination).


A
SphI
0.5 Kb
0.5 Kb
pre-mir-34a
Neo
Thymidine Kinase
pre-mir-34a
Neo
B
1
2
Wild-type allele
Targeted allele
C
1
2
Targeted allele
Wild-type allele

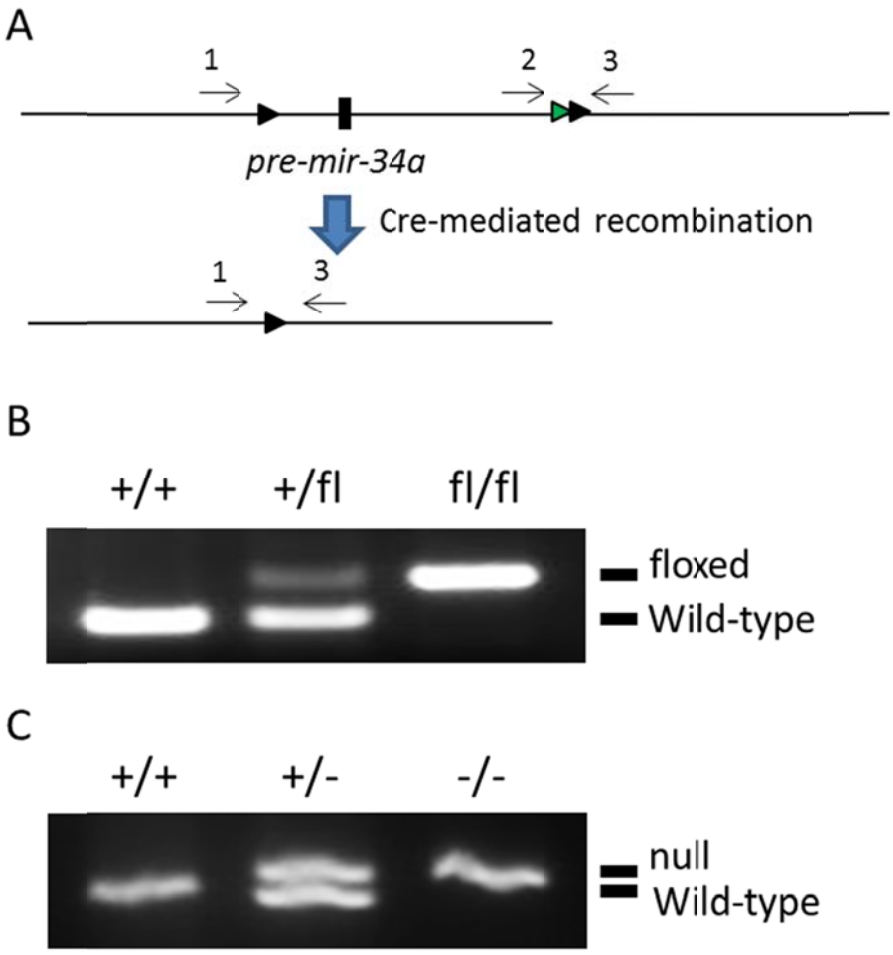


Figure 4.4 Genotyping of mice carrying conditional *mir-34a* allele and *mir-34a* null allele. A. Schematic diagram of conditional *mir-34a* allele and *mir-34a* null allele. Black and green arrowheads indicate *loxP* and *FRT* sites. Black arrows indicate genotyping PCR primer region. B. DNA from wild-type, heterozygous and homozygous mice of conditional *mir-34a* allele was subjected to PCR genotyping. 293 bp band indicates the allele flanked by loxP sites (floxed) allele, and 196 bp wild-type allele. C. DNA from wild-type, heterozygous and homozygous mice of *mir-34a* knock-out allele was subjected to PCR genotyping. 216 bp band indicates null allele, and 196 bp wild-type allele.

Table 4.1 Genotypes of mice resulting from crosses between *mir-34a*$^{-/-}$*mir-34b/c*$^{+/-}$ and *mir-34a*$^{-/-}$ *mir-34b/c*$^{+/-}$ mice

Genotypes	% Expected frequency	% Frequency (n)
mir-34a$^{-/-}$ *mir-34b/c*$^{+/+}$	25 %	25 %(n=8)
mir-34a$^{-/-}$ *mir-34b/c*$^{+/-}$	50 %	47 % (n=15)
mir-34a$^{-/-}$ *mir-34b/c*$^{-/-}$	25 %	28 % (n=9)
Total	100%	100 % (n=32)

Table 4.2 Genotypes of mice resulting from crosses between *mir-34a*$^{+/-}$ *mir-34b/c*$^{-/-}$and *mir-34a*$^{-/-}$ *mir-34b/c*$^{+/-}$ mice

Genotypes	% Expected frequency	% Frequency
mir-34a$^{+/-}$ *mir-34b/c*$^{+/-}$	25 %	24 % (n=7)
mir-34a$^{-/-}$ *mir-34b/c*$^{+/-}$	25 %	24 % (n=7)
mir-34a$^{+/-}$ *mir-34b/c*$^{-/-}$	25 %	31 % (n=9)
mir-34a$^{-/-}$ *mir-34b/c*$^{-/-}$	25 %	21 % (n=6)
Total	100 %	100 % (n=29)

Table 4.3 Genotypes of mice resulting from crosses between *mir-34*$^{-/-}$ *mir-34b/c*$^{-/-}$ with *mir-34a*$^{-/-}$ *mir-34b/c*$^{+/-}$ mice

Genotypes	% Expected frequency	% Frequency
mir-34a$^{-/-}$ *mir-34b/c*$^{+/-}$	50 %	45 % (n=5)
mir-34a$^{-/-}$ *mir-34b/c*$^{-/-}$	50 %	55 % (n=6)
Total	100 %	100 % (n=11)

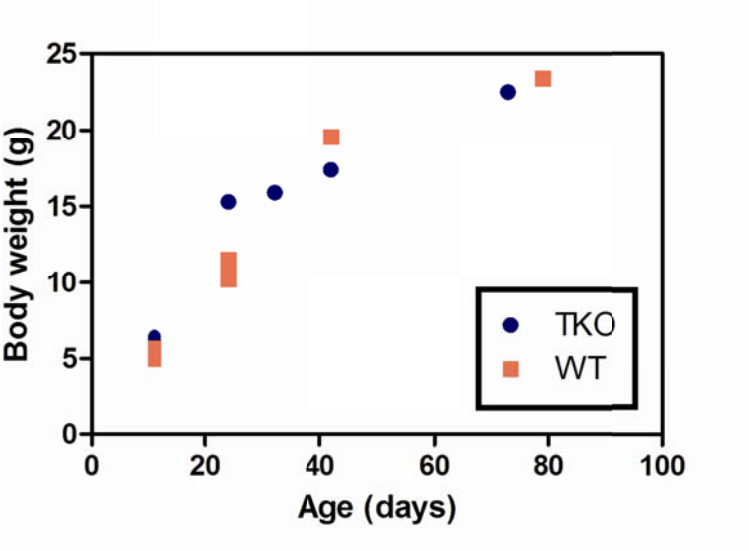


Figure 4.5 Body weights of wild-type (WT) and *mir-34a*$^{-/-}$*mir-34b/c*$^{-/-}$ (TKO) female mice.

um (OSE) were isolated from *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ and wild-type mice, and miR-34 family expression level was confirmed (Figure 4.6). As expected, expression of MET, one of the shared targets of miR-34 family was increased in *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$OSE compared to wild-type OSE (described in Chapter 3, Figure 3.7B).

Unlike *p53*$^{-/-}$ mice, *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ did not develop hematopoietic malignancies, such as lymphoma by 6 months of age. In order to explore any postnatal defects in *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ (triple knock-out, TKO) mice, one 3 weeks-old and two 9 weeks-old female TKO mice were sacrificed together with age- and sex-matched wild-type mice of similar genetic background.

During necropsy, it was noted that TKO mice had significantly reduced sizes of spleens and thymuses by 9 weeks of age (Figures 4.7 A and B and 4.8 A and B). In 3 weeks old TKO mice, there was no histologically detectable difference in the spleen, as compared to age-matched wild-type (Figure 4.7C and D). To the contrary, 9 weeks old TKO mice showed hypoplasia of white pulp and splenic atrophy compared to wild-type mice (Figure 4. 7A, B, E and F). Similarly, the thymuses from 3 weeks old wild-type and TKO mice had no histologically detectable differences (Figure 4. 8C and D). However, in 9 weeks old TKO mice, the overall size of thymus was smaller than that of wild-type (Figure 4. 8A and B), and the cortex region of the thymus was not distinguishable from the medulla region, while wild-type TKO mice had clear demarcation between the cortex and the medulla regions (Figure 4. 8E and F). This indicates that the thymus in TKO mice undergoes accelerated aplasia as compared to wild-type mice during postnatal development. The forestomach of both 9 weeks old TKO mice showed hyperplasia and ulceration of the squamous epithelium (Figure 4. 9A and B). In addition, focal hyperplasia of Purkinje neurons was detected in the cerebellum of one of two 9 weeks old TKO mice (Figure 4. 9C and D).

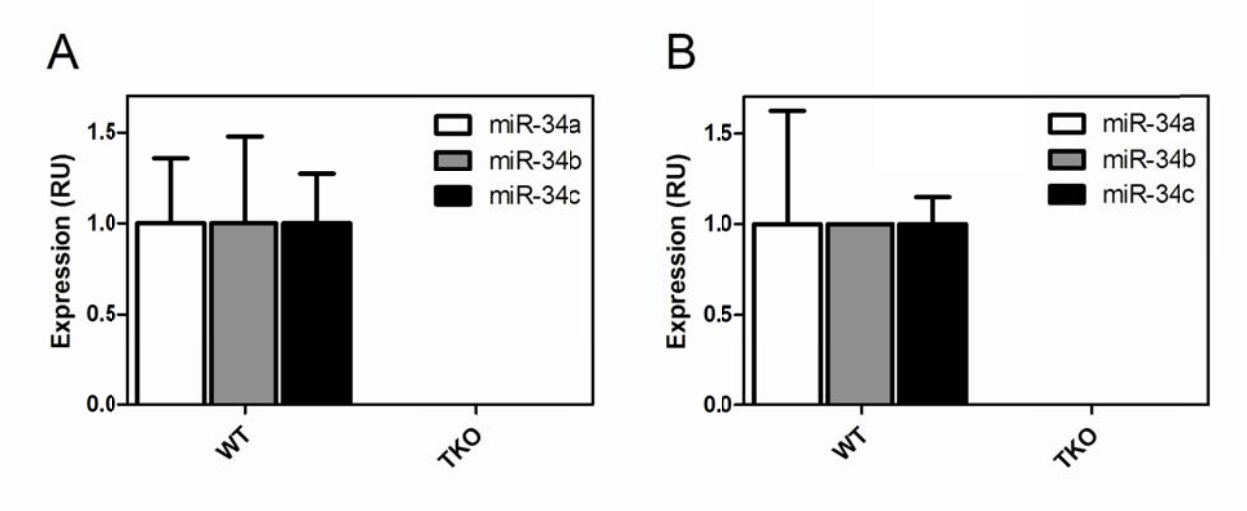


Figure 4.6 miR-34 expression in the brain (A) and the ovarian surface epithelium (OSE, B) of wild-type (WT) and *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ (TKO) mice. Levels of individual miR-34 family member expression (average ± SD, n= 3) were measured by qRT-PCR and normalized to RNU6B (RU).

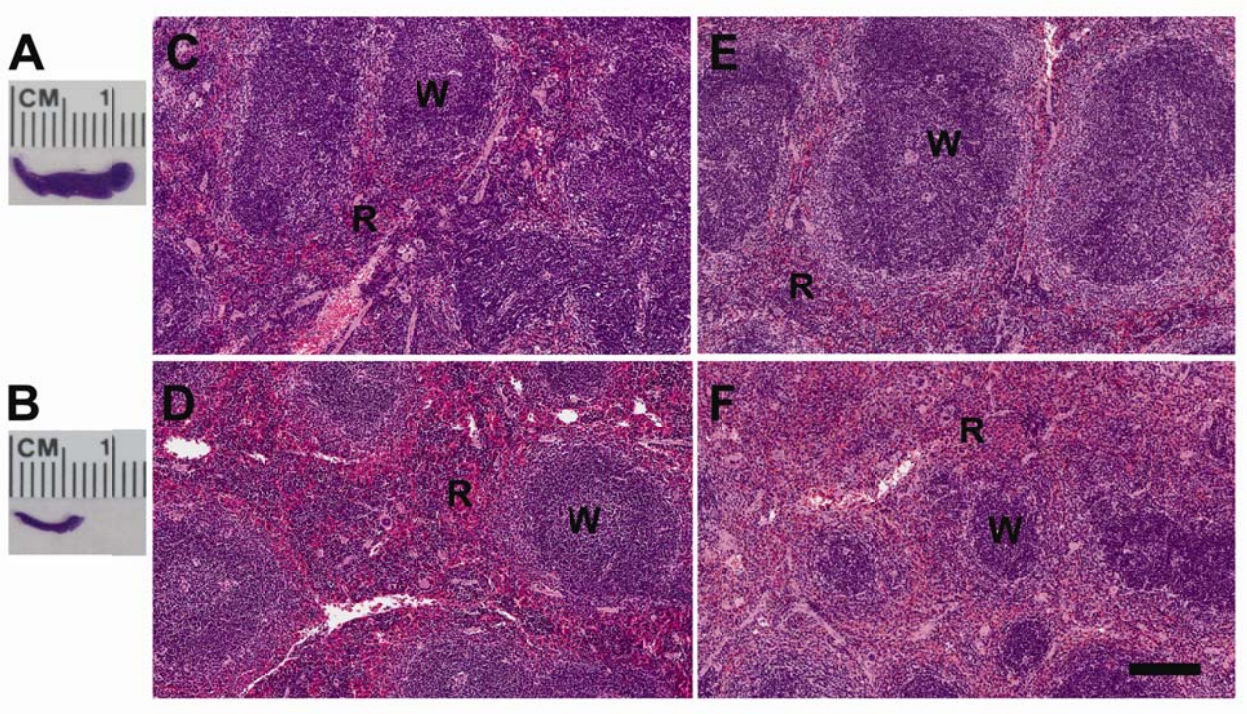


Figure 4.7 Morphological changes in the spleen of *mir-34a*$^{-/-}$*mir-34b/c*$^{-/-}$ mice (TKO). Histology of the spleens of 3 weeks (C and D) and 9 weeks old (A, B, E, and F) wild-type (A, C and E) and TKO (B, D and F) mice. Hematoxylin and eosin staining. W, white pulp; R, red pulp. Scale bar = 150 μm.

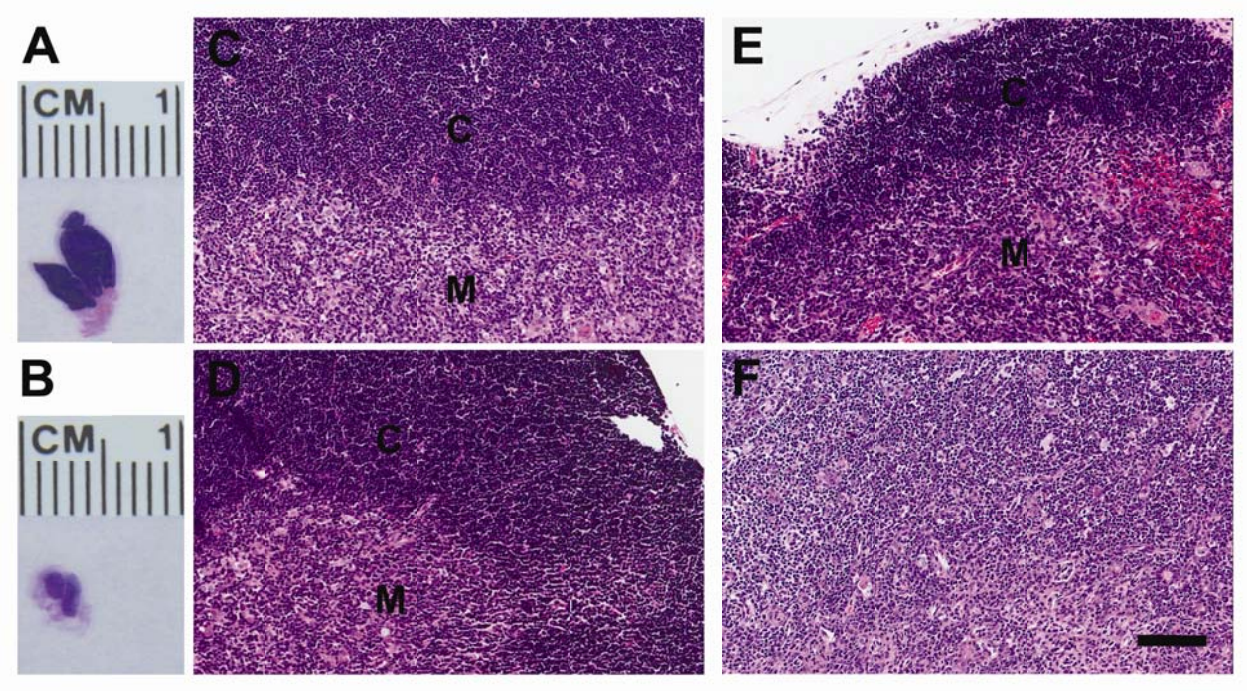


Figure 4.8 Morphological changes in the thymus of *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ mice (TKO). Histology of the thymuses of 3 weeks (C and D) and 9 weeks old (A, B, E and F) wild-type (A, C and E) and TKO (B, D and F) mice. Hematoxylin and eosin staining. C, cortex; M, medulla. Scale bar = 100 μm.

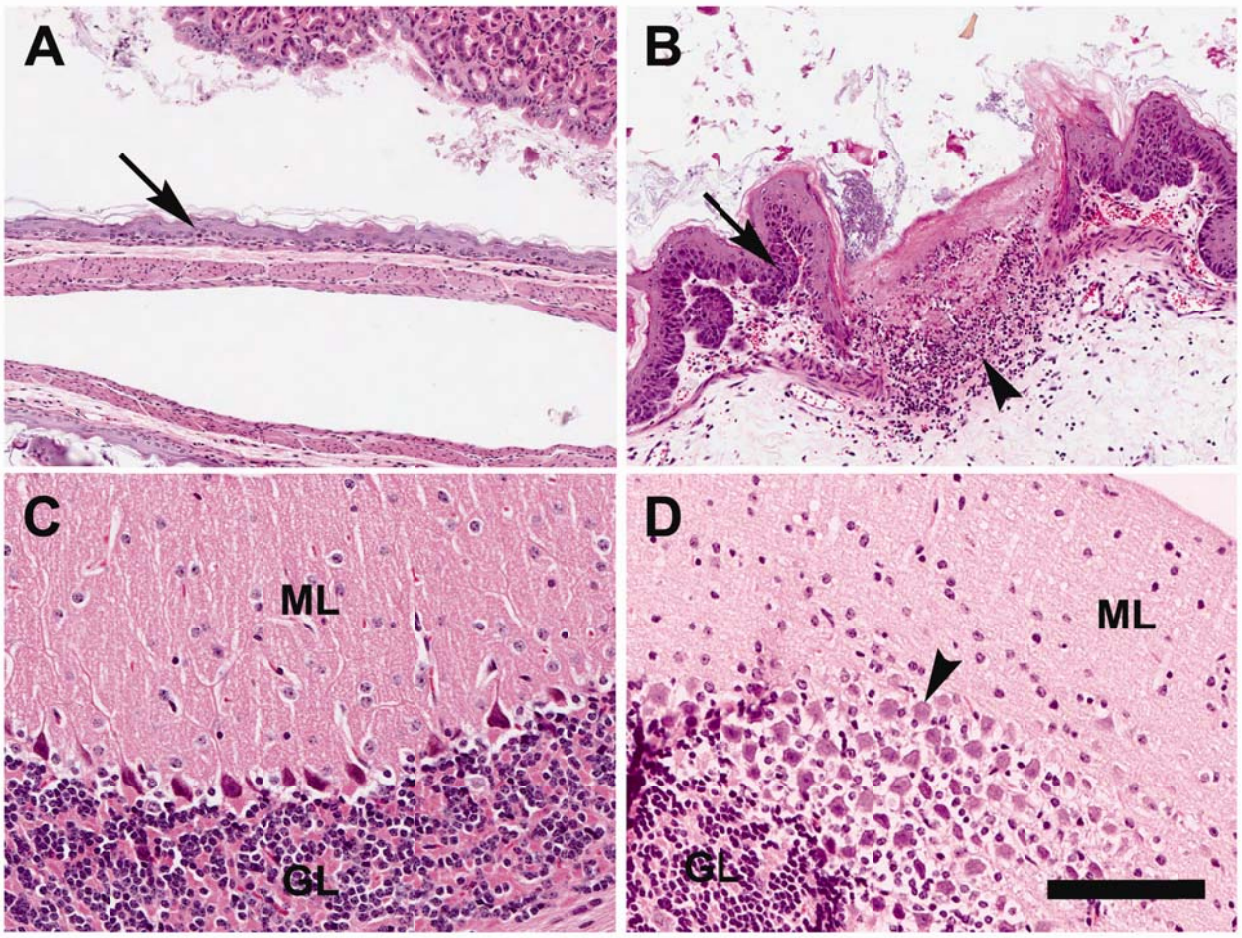


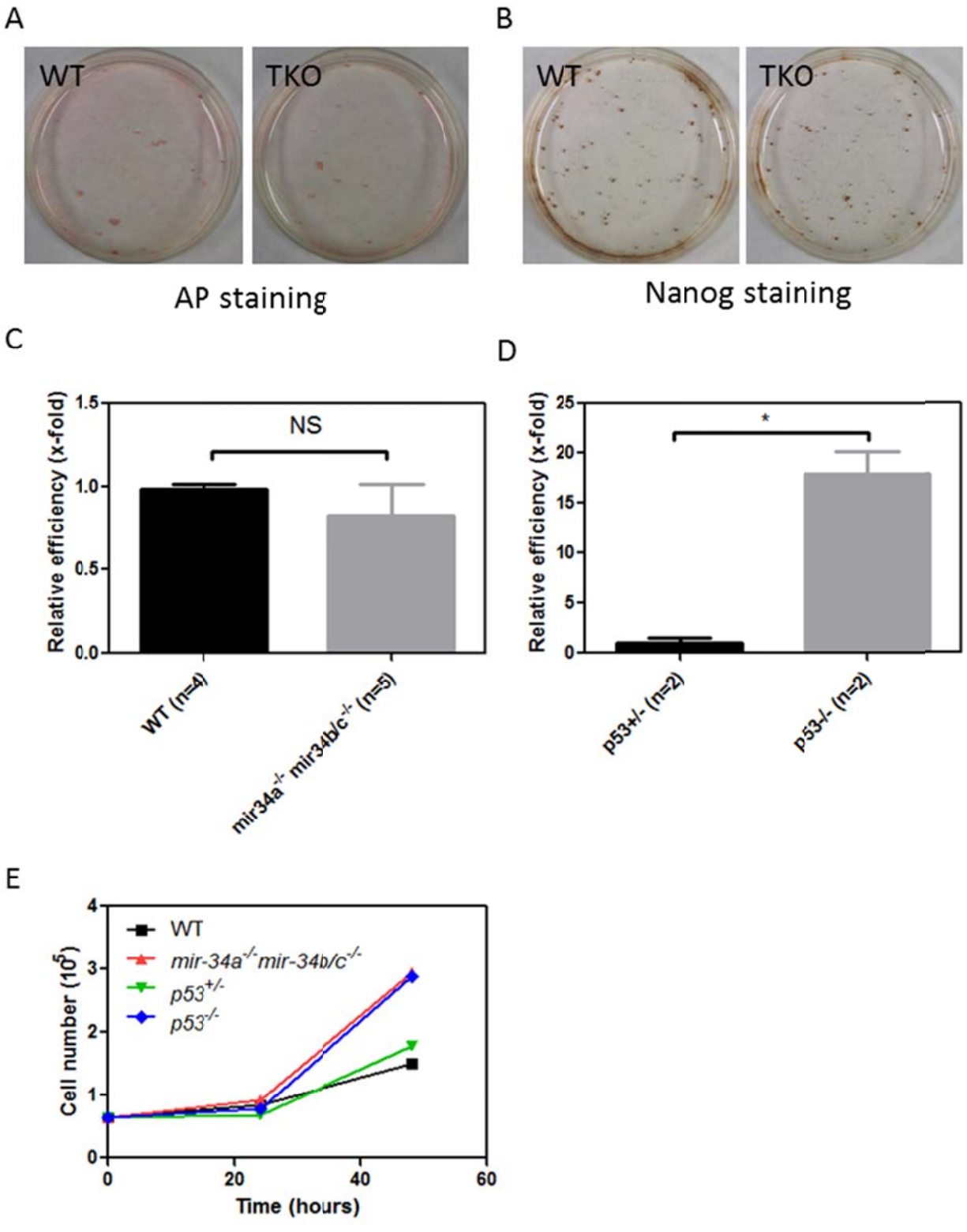
Figure 4.9 Morphological changes in the stomach and the cerebellum of *mir-34a*$^{-/-}$*mir-34b/c*$^{-/-}$ mice (TKO). (A-B) Forestomach of 9 weeks old wild-type (A) and TKO (B) mouse. Note ulceration with inflammatory infiltrates (arrowhead) and hyperplasia of squamous epithelium (arrow) in the forestomach of TKO mouse compared to normal squamous epithelium (arrow) in wild-type. (C-D) Cerebellum in 9 weeks old wild-type (C) and TKO (D) mouse. Note focal hyperplasia of Purkinje cells in (arrowhead) TKO mouse. ML, molecular layer; GL, granule cell layer. Scale bar is 200 μm (A and B), 100μm (C and D).

4.4.4 iPS cell reprogramming

Loss of p53 has been shown to facilitate iPS cell reprogramming efficiency (Hanna et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009a; Marion et al., 2009; Utikal et al., 2009). Furthermore, c-Myc and Klf4 are among downstream targets of miR-34 (Christoffersen et al., 2010; Kong et al., 2008; Lujambio et al., 2008 and our unpublished observations). Thus, we hypothesized that *mir-34* family inactivation may recapitulate loss of p53 in iPS cell reprogramming at least in part. First, we isolated TTFs from TKO and age-matched wild-type mice, and subjected to them to iPS cell reprogramming with 4 (Oct-4, Klf-4, Sox-2 and c-Myc) and 3 (Oct-4, Klf-4 and Sox-2) original Yamanaka factors (Takahashi and Yamanaka, 2006), followed by assessment of AP and Nanog staining (Figure 4.10A-C). iPS cell reprogramming with 4 factors resulted in no significant difference in AP and Nanog staining between wild-type and TKO (Figure 4. 10A-C), while iPS cell reprogramming with 3 factors generated 2 and 4 AP positive clones in wild-type and TKO TTFs respectively. To rule out the possibility that TTFs may behave differently from MEFs in reprogramming process, TTFs from heterozygous and homozygous p53 deficient mice were isolated and subjected to iPS cell reprogramming. Consistent with previous findings, loss of p53 dramatically improved reprogramming efficiency along with increased proliferation in TTFs (Figure 4. 10D and E). Notably, *mir-34* deficiency caused increased proliferation rate similar to that resulting from *p53* inactivation. These findings indicate that p53 effects on iPS cell reprogramming are not limited to accelerated proliferation (Figure 4.10A-C and E).

Since TTFs have a less replicative potential than MEFs, we further explored the iPS cell reprogramming efficiency with MEFs. In addition, to reduce technical biases from different MEFs preparation and strain backgrounds, MEFs from *mir-34a*$^{loxP/loxP}$*mir-34b/c*$^{loxP/loxP}$ were infected with

Figure 4.10 iPS cell reprogramming efficiency of TTFs from wild-type and TKO. A and B. TTFs from TKO and age-matched wild-type mice were isolated, subjected to iPS cell reprogramming with Oct-4, Sox-2, Klf-4 and c-Myc and stained with AP and Nanog respectively. C. Relative iPS cell reprogramming efficiency from three independent experiments was quantified. D. TTFs from heterozygous and homozygous p53 deficient mice were used for iPS cell reprogramming. p53 deficiency increases iPS cell reprogramming efficiency (P=0.01). Relative iPS cell reprogramming was quantified based on AP-positive colony numbers compared to control experiments. E. Cell growth curves after induction of iPS cell reprogramming factors by doxycycline. Note similar growth rates between *p53* and *mir-34* deficiency. Bars, SD. iPS cell reprogramming, AP staining and cell growth analysis were performed by Christian Abratte, Cornell Induced Pluripotent Stem Cell Core Facility.


A
WT
TKO
AP staining
B
WT
TKO
Nanog staining
C
Relative efficiency (x-fold)
1.5
1.0
0.5
0.0
NS
WT (n=4)
mir34a-/- mir34b/c-/- (n=5)
D
Relative efficiency (x-fold)
25
20
15
10
5
0
*
p53+/- (n=2)
p53-/- (n=2)
E
Cell number (10^5)
4
3
2
1
0
WT
mir-34a-/-mir-34b/c-/-
p53+/-
p53-/-
0
20
40
60
Time (hours)

either Ad-Blank or Ad-*Cre*, and subjected to iPS cell reprogramming with four factors. Consistent with TTFs iPS cell reprogramming results, there was no statistically significant difference between wild-type and *mir-34* null MEFs (Fig. 4.11).

4.4.5 miR-34a expression and DNA damage response

In order to explore potential involvement of miR-34a in response to IR, wild-type mice were exposed to 15 Gy and subjected to histological analysis (Figure 4. 12) and miR-34a *in situ* hybridization (Figure 4.13). 1, 3, and 5 days after irradiation. Consistent with previous studies (Komarova et al., 2004; Martin et al., 1998), in the small intestine, the size and number of intestinal crypts was markedly decreased 3 days after IR (Figure 4. 12C and G) and significant destruction of villi was observed in 5 days after IR (Figure 4.12D and H). At the same time, intestinal crypts of the large intestine were less damaged as compared to the small intestine (Figure 4. 12J-L). On day 5, hyperplastic regenerative crypts were observed both in small and large intestine (Figure 4. 12D, H and L). Differential responses from small and large intestine after 15 Gy of IR are consistent with previous reports that large intestine is less sensitive than small intestine in DNA damage response (Hong et al., 2005; Merritt et al., 1997; Potten et al., 1997).

Next, we investigated miR-34a expression in the small and large intestine after 15 Gy of IR (Figure 4. 13). While miR-34a was not detectable in the large intestine without IR, miR-34a was expressed in both villi and crypts in the proximal and distal part of small intestine (Figure 4. 13A, E and I). miR-34a expression was found mainly in the crypts of all intestinal regions one day after IR (Figure 4. 13B, F and J). Along with decreased number of small intestinal crypts, the levels of miR-34a were markedly reduced 3 days after IR, particularly in the small intestine

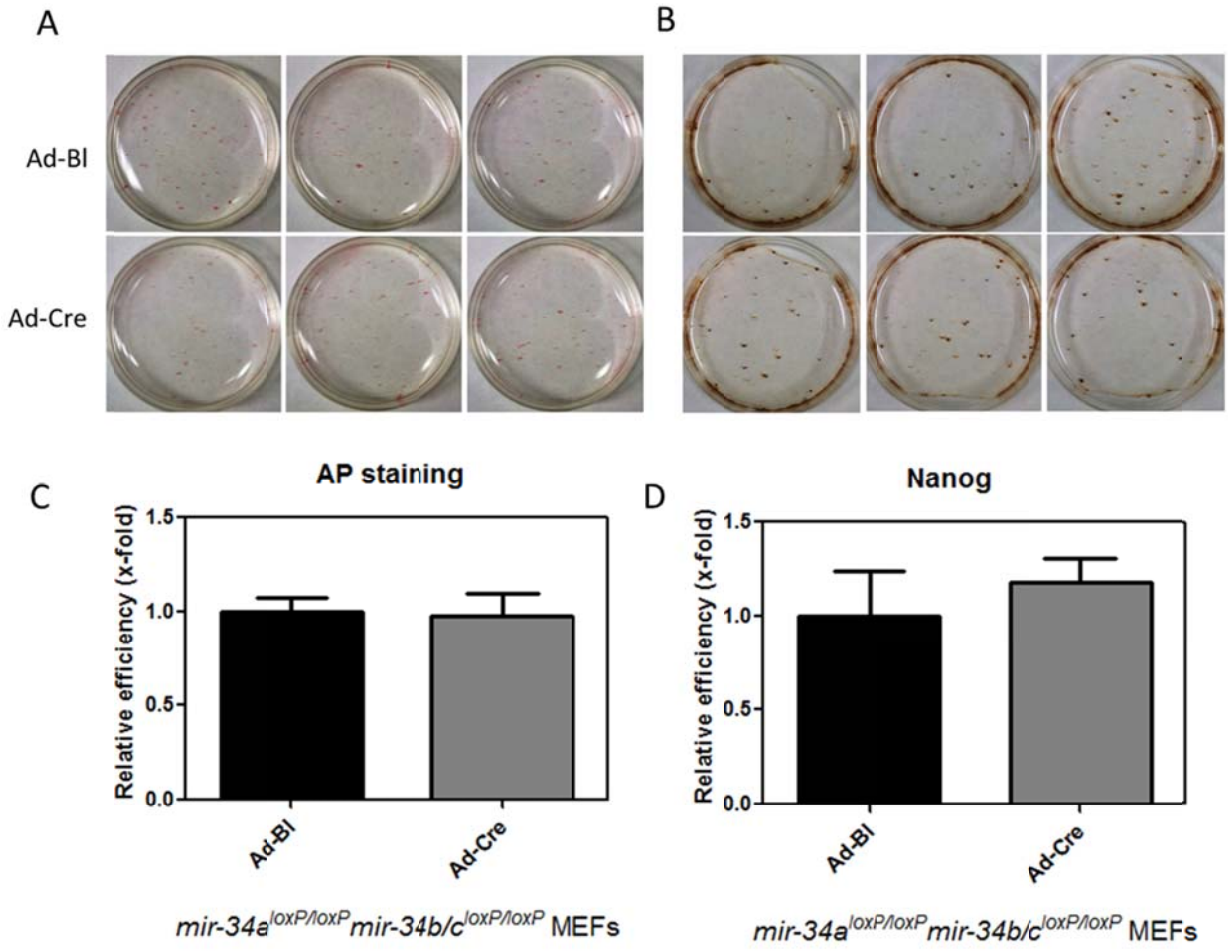


Figure 4.11 iPS cell reprogramming efficiency of mouse embryonic fibroblasts (MEFs) from *mir-34a*$^{loxP/loxP}$*mir-34b/c*$^{loxP/loxP}$ after Ad-Bl or Ad-*Cre* infection. A and B. *mir-34* inactivated MEFs and control MEFs were subjected to iPS cell reprogramming with Oct-4, Sox-2, Klf-4 and c-Myc and stained with AP and Nanog respectively. C and D. Relative iPS cell reprogramming efficiency from triplicate experiments was quantified based on AP and Nanog positivity respectively. Bars, SD. iPS cell reprogramming and AP staining were performed by Christian Abratte, Cornell Induced Pluripotent Stem Cell Core Facility.

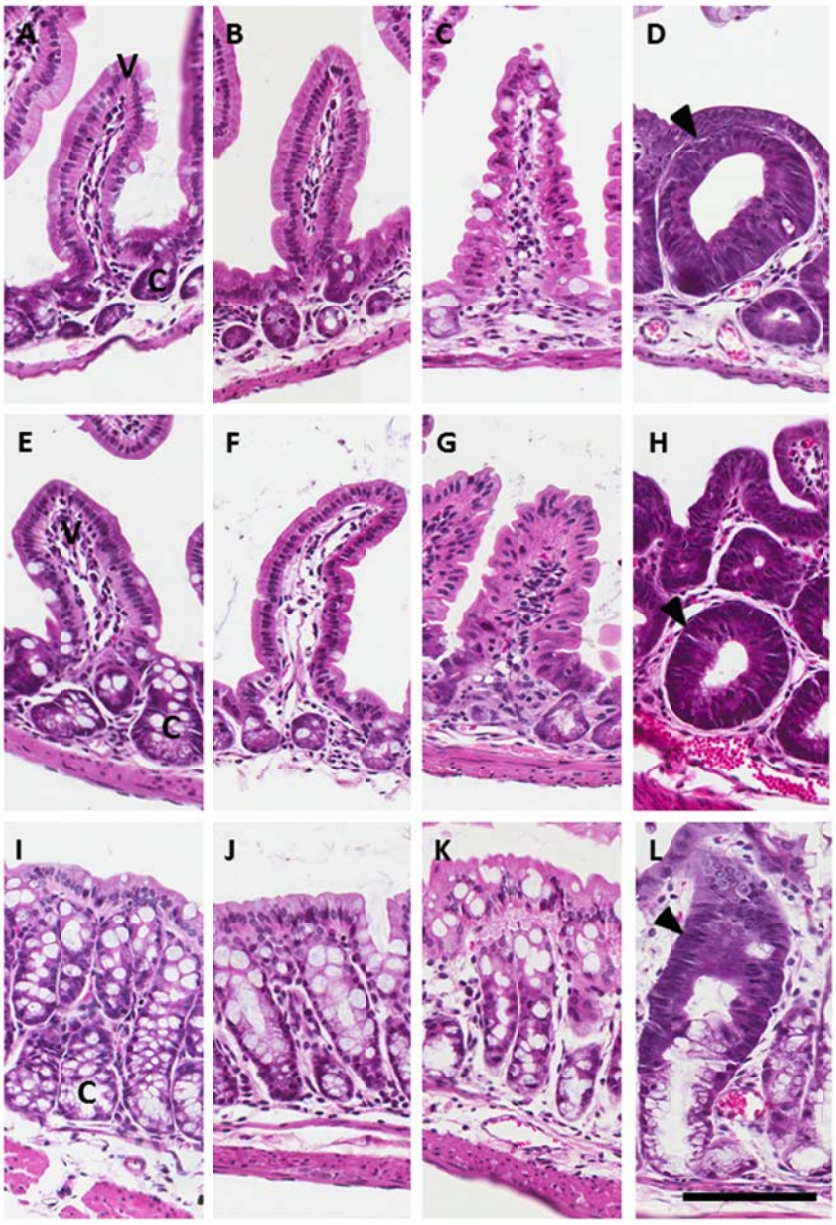


Figure 4.12 H&E staining of small and large intestine after 15 Gy IR. (A-D) The proximal part of small intestine, (E-H) the distal part of small intestine, and (I-L) the large intestine before (A, E, and I) and 1 day (B, F, J), 3 days (C,G, K) and 5 days after 15 Gy IR (D, H, L). V, intestinal villi; C, intestinal crypt; Arrowheads, hyperplastic regenerative foci. Hematoxylin and eosin, Scale bar is 50 μm for all images.

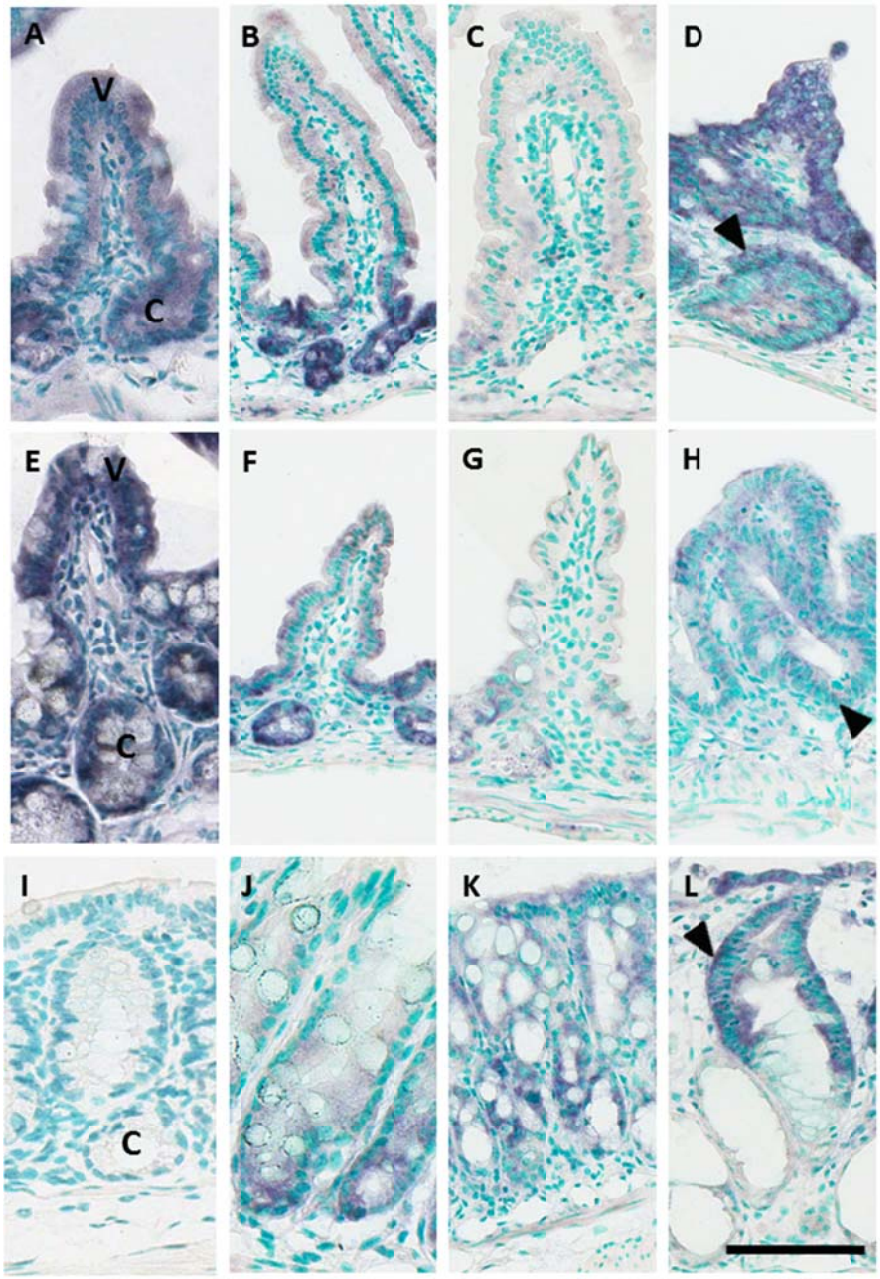


Figure 4.13 miR-34a *in situ* hybridization of small and large intestine after 15 Gy IR. (A-D) The proximal part of small intestine, (E-H) the distal part of small intestine, and (I-L) Large intestine, before (A, E, I), and 1 day after 15 Gy IR (B, F, J), 3 days after 15 Gy IR (C, G, K), and 5 days after 15 Gy IR (D, H, L). Methyl green counterstaining. V, intestinal villi; C, intestinal crypt; arrowheads, hyperplastic regenerative foci. Scale bar is 50 μm for all images.

(Figure 4. 13C and G). However, as regenerative crypts appeared 5 days after IR, miR-34a levels became high in hyperplastic enterocytes (Figure 4. 13D, H and L). Notably, miR-34a expressing cells tended to move towards villi (Figure 4. 13D and H). Overall pattern of miR-34a expression, with preferential presence of miR-34a in the crypt region after IR was similar in small and large intestine, but the number of intestinal crypts in the large intestine was not decreased 3 days after IR.

Given the expression pattern of miR-34a in GI tract after IR, we hypothesized that miR-34 may play a role in DNA damage response to IR. To initiate exploration of this role, 4 weeks old *miR-34b/c*$^{-/-}$ female mice and their wild-type littermates were subjected to 10 Gy (hematopoietic syndrome) and 15 Gy (gastrointestinal tract syndrome) of IR. While 10 Gy dose exposure did not show any difference in survival between wild-type and *mir-34b/c*$^{-/-}$ mice (Figure 4.14A), *mir-34b/c*$^{-/-}$ mice showed IR-resistant phenotype after 15 Gy IR exposure (Figure 4.14B).

4.5 Discussion

In order to study the role of miR-34 in development and carcinogenesis, we have prepared mice with both conventional and conditional inactivation of *mir-34*. While mice completely lacking miR-34 family are alive, conventional knockout approach has several potential shortcomings. Among those are potential abnormalities during postnatal development and ageing which may make it impossible to study gene function in the cell type of choice. Furthermore, non-physiological adaptive responses from a specific gene knock-out may obscure the study of gene function (Williams and Wagner, 2000).

One of major limitations for generation of conditional knockout mice is the difficulty and

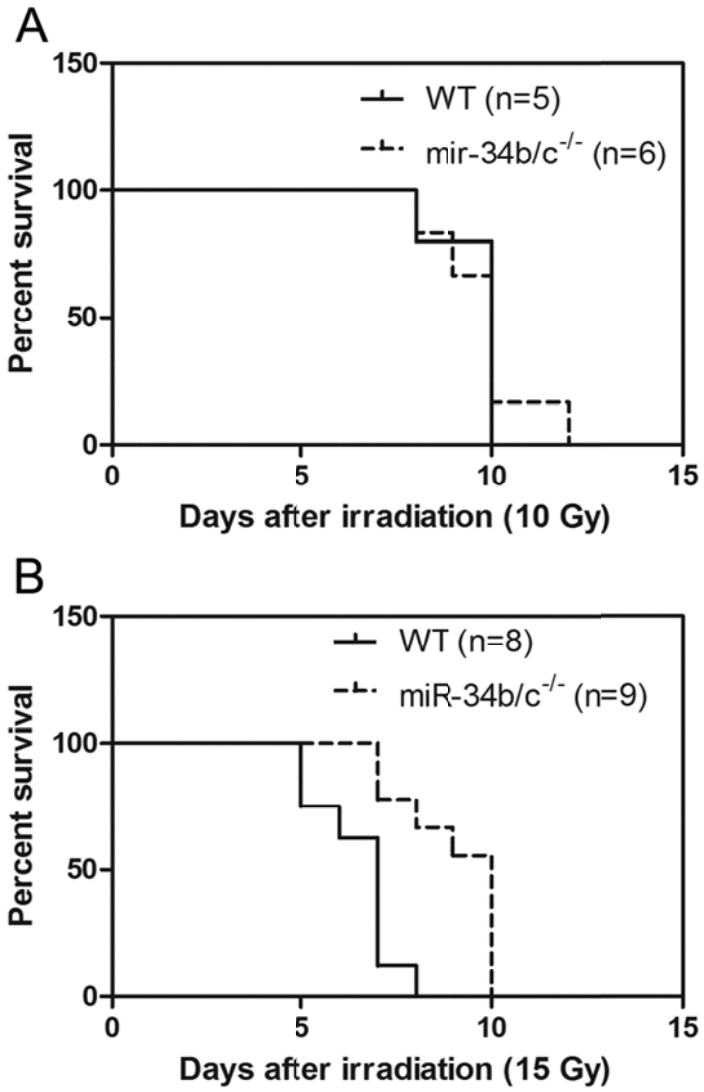


Figure 4.14 Survival analysis of 10 Gy (A) and 15 Gy (B) whole body exposure of IR in wild-type and *mir-34b/c*$^{-/-}$mice. A. Median survival for wild-type (10 days) and *miR-34b/c*$^{-/-}$ (10days) was similar (log-rank test, P=0.8801), B. Difference of median survival for wild-type (7 days) and *miR-34b/c*$^{-/-}$ (10 days) was statistically significant (log-rank test, P=0.0012).

time it takes to make a targeting vector. To address this problem we have taken advantage of efficient homologous recombination performed of phage-encoded proteins in *E.coli* (Liu et al., 2003). By using proteins encoded by the *Red* genes of bacteriophage λ, we inserted linear double-strand DNA fragments into DNA cloned on plasmids or bacterial artificial chromosomes (BACs) by homologous recombination. The resulting targeting constructs carrying *loxP* sites in two introns were introduced into to mouse ES cells by standard homologous recombination. Cre recombinase expression driven by tissue specific promoter or drug-inducible system allows a gene to be inactivated in spatio-temporal fashion by Cre-*loxP*-mediated recombination (Nagy, 2000). Therefore, conditional *mir-34* knockout mice will allow us to study precise role of miR-34 in physiologically relevant settings in cell culture and *in vivo*.

As a direct target of p53, miR-34 family plays a role in cell cycle arrest, apoptosis, senescence, cell migration and invasion (Hermeking, 2010). However, until generation of mice deficient for all miR-34 family it has been impossible to accurately establish p53-dependent and independent functions of these miRNAs.

Our preliminary histology analysis of mice null for all *mir-34* genes suggests that miR-34 is involved in development of such organs of the hematopoietic system as the thymus and spleen, although further careful examination is required. These findings are of particular interest because ectopic expression of miR-34a has been shown to affect B cell and T cell development (Rao et al., 2010; Stadanlick et al., 2011). Specifically, Rao et al. and Stadanlick et al. have shown that constitutive miR-34a expression blocks B cell development at the pro-B-cell-to-pre-B-cell transition and arrest T cell development, respectively. Notably, *Dicer* deficiency is also known to lead to differential T cell lineage choice and defect in invariant natural killer T cells development (Cobb et al., 2005; Muljo et al., 2005; Seo et al., 2010). Thus it would be interesting to test if *mir-34* deficiency contributes to this phenotype of *Dicer* deficiency.

Focal hyperplasia of Purkinje cells in cerebellum also raise a possibility that miR-34 plays a role in cerebellar development. Given tissue specific expression pattern of miR-34 in Purkinje neurons (Corney, 2010), hyperplasia of Purkinje cells may result from miR-34 deficiency. Interestingly, such Purkinje neuron hyperplasia has been reported in *Bcl-2* transgenic mice (Zanjani et al., 1996). Bcl-2 has anti-apoptotic functions, playing a critical role in determining cell survival and death (Kelly and Strasser, 2011), and also one of the miR-34 targets (Ji et al., 2009). It is known that mouse Purkinje cells express Bcl-2 from E 18.5 until the first postnatal month, and then expression of Bcl-2 declines substantially by 5 months (Merry et al., 1994). Thus, it is tempting to speculate that *mir-34* deficiency results in increased Bcl-2 expression, and subsequently causes hyperplasia of Purkinje cells similar to phenotype of *Bcl-2* transgenic mice. Since *Bcl-2* transgenic mice exhibited decreased RotaRod performance despite increased number of Purkinje cells (Goswami et al., 2005), it would be interesting whether *mir-34* deficiency causes any defects related to motor neuron disease.

Ulceration of forestomach and hyperplasia of the surrounding gastric squamous epithelium in TKO mice raises a possibility that *mir-34* deficiency may cause imbalance between gastric epithelial cell proliferation and death in maintaining gastric mucosal integrity. Since miR-34 also involves in anti-proliferative function, it is possible that loss of *mir-34* alters the balance between proliferation and apoptosis toward proliferative, resulting in hyperplasia and gastric ulcer. Alternatively *mir-34* deficiency may cause inflammation and/or a defect in epithelial regeneration, followed by hyperplasia. Interestingly, p63 deficiency has been shown to result in metaplasia of the squamocolumnar junction (Wang et al., 2011). At the same time, p63 also has been known to regulate miR-34a expression (Su et al., 2010). Since esophageal and gastric adenocarcinomas are associated with chronic inflammation and are preceded by robust metaplasia, it is of interest whether this is connected to esophageal or gastric cancer development.

By 6 month of age no overt malignant lesions have been detected by us in mice lacking all miR-34 members. Since the majority of epithelial cancers develop by 1-1.5 years of age long-term experiments should clarify if miR-34 plays an important role in cancer initiation. However, lack of hematopoietic malignancies, such as leukemias and lymphomas, typical for p53 null mice indicates that tumor suppressive functions of miR-34 and p53 are quite different.

Based on our observations that miR-34a expression was downregulated regardless of *p53* mutational status in ovarian cancers (described in Chapter 2), it is also possible that loss of miR-34 may cooperate with *p53* mutation in carcinogenesis, albeit miR-34 is a downstream in p53 signaling. Furthermore, as we describe in Chapter 3, deficiency for p53 and miR-34 cooperate in elevation of MET expression resulting in increased cell invasion. Whether miR-34 loss combined with p53 loss increases immortalization process in cell culture system and tumor susceptibility *in vivo* would be among our next questions to be addressed.

Since iPS cells were generated from somatic cells by Yamanaka group (Takahashi and Yamanaka, 2006), a number of studies aimed to improve reprogramming efficiency, to reduce required factors, to utilize easily accessible cells as a source and to ensure safety issues, such as malignant transformation, have been reported (Gonzalez et al., 2011). A number of groups showed that loss of p53 accelerates reprogramming process (Hanna et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009a; Marion et al., 2009; Utikal et al., 2009). Hanna et al. proposed two different pathways to improve iPS cell reprogramming efficiency via proliferation-dependent and –independent way. While Nanog expression facilitates iPS cell reprogramming process itself in proliferation-independent manner, the effect of p53 loss was largely proliferation-dependent (Hanna et al., 2009). However, it is still possible that p53 may affect iPS cell reprogramming process itself, since p53 is also known to regulate stem cell differentiation and stemness gene expression such as Oct-4 and Nanog (Lin et al., 2005; Qin et al., 2007). According to literature reports (Christoffersen et al., 2010; Kong et al., 2008;

Lujambio et al., 2008) and our unpublished data (Hwang et al.), miR-34 family is able to target c-Myc and Klf-4, two of the four initially identified reprogramming factors. We reasoned that *mir-34* inactivation may lead to increased expression of c-Myc and Klf-4 thereby resulting in improved iPS cell reprogramming efficiency. However we did not observe the anticipated effect. Unlike p53 deficiency, *mir-34* deficiency did not improve reprogramming of TTFs. Notably, TTFs deficient for with p53 or all miR-34 has similarly increased proliferation rate suggest that increased proliferation is not the only mechanism by which p53 deficiency improves reprogramming efficiency. Our observations that *mir-34* null MEFs or OSE were not easily immortalized in cell culture (Hwang et al., unpublished) indicate that unlike p53 deficiency, *mir-34* inactivation may not be sufficient to overcome the senescence barrier. It remains to be determined if elimination of senescence barrier is the only additional mechanism by p53 counteracts reprograming. It is also remains to be established if introduction of miR-34 to cells undergoing reprogramming may attenuate this process.

p53 activation by DNA damage such as IR leads to apoptosis, cell cycle arrest and senescence, although responses are highly cell-type dependent and dose-dependent (Gudkov and Komarova, 2010). In the mouse, 10 Gy of IR causes hematopoietic syndrome. The deleterious effects of this syndrome are largely mediated by p53 because p53 deficiency results in extended mouse survival. On the contrary, high dose of IR (>15 Gy), which results in gastrointestinal (GI) tract syndrome associated with shorter survival of p53 deficient mice, indicating the radioprotective role of p53 (Komarova et al., 2004). Komarova et al. proposed that the balance of two functions, growth arrest and apoptosis induced by p53 may determine radiation-induced injury and its final outcome (Komarova et al., 2004), albeit it remains unclear how p53 differentially responds in different tissues and doses, and which downstream targets of p53 are responsible for these outcomes. As a p53 downstream target gene, p21 deficiency has been shown to have a similar radiosensitive phenotype of p53 deficiency in 15 Gy IR (Komarova

et al., 2004). At the same time, loss of PUMA, another target of p53 has been shown to induce radioresistant phenotype in GI tract syndrome (Qiu et al., 2008). Therefore, the effect of downstream targets of p53 may be different depending on its specific role in tissues.

Our findings of differential expression pattern of miR-34a in the small and large intestine after 15 Gy whole body exposures of IR, are in agreement with previous observations that the small intestine is more sensitive than large intestine after 15 Gy IR (Potten et al., 1997). In the small intestine we observed that miR-34a expressing cells were almost depleted 3 days after IR, likely due to IR-induced apoptosis and cell death. Notably, regenerating crypts highly expressed miR-34a 5 days after IR. In the case of large intestine, miR-34a expressed in the crypt region 1 day after IR, and miR-34a expressing cells seemed to move toward villi without depletion of miR-34a expressing cells. Interestingly, as opposed to miR-34a expression pattern, Bcl-2 is expressed in the large intestine crypt location of stem cells but not in that of small intestine (Merritt et al., 1997). In addition, differential expression pattern of Bcl-2 in the small and large intestine has been proposed as the reason for differential ability of these regions to induce apoptosis after IR (Potten et al., 1997). Thus, it is also possible that differential miR-34a expression pattern may account for differential sensitivity to induce apoptosis after IR. Accordingly, our finding that *mir-34b/c* knock-out mice exhibited radioresistance in 15 Gy but not 10 Gy, as compared to littermate wild-type mice, may be due to increased Bcl-2 expression resulting from *mir-34b/c* inactivation. Unfortunately, our *in situ* hybridization failed to detect miR-34b/c in GI tract so far, consistent with overall low levels of expression of these miRNAs in the intestinal epithelium with miR-34b/c GI tract expression being reported only in 14.5 embryo (Diez-Roux et al., 2011). We are currently evaluating DNA damage response in *mir-34a*$^{-/-}$ *and mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ mice. These studies, together with identification of involved downstream targets, should determine the role of miR-34 pathogenesis of DNA damage response in the GI tract syndrome.

Taken together, we report the initial characterization of *mir-34* deficient mice including defects in hematopoietic system development, focal hyperplasia of Pukinje neurons and gastric ulceration. Failure of *mir-34* loss to recapitulate the p53 loss phenotype in iPS cell reprogramming may provide a clue for the proliferation-independent mechanism of p53 in iPS cell reprogramming process. Further studies on DNA damage response and detailed characterization of newly developed *mir-34* conditional and conventional knock-out mice will reveal the role of miR-34 in development, DNA damage response and carcinogenesis.

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CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

5.1 Summary

Previous observations by us and others have shown that *p53* transcriptionally activates genes encoding for miR-34 family. Furthermore, it has been reported that miR-34 expression is frequently downregulated in several types of cancer. Thus, we hypothesized that miR-34 family expression would be affected in high grade serous ovarian adenocarcinomas, particularly since *p53* mutation is the most common genetic alteration in this type of cancer. Indeed we found that miR-34 family expression is frequently downregulated in EOC. Additionally, we have further identified epigenetic inactivation or copy number variations of miR-34 family and found that miR-34b/c is associated with disease progression. Interestingly, while downregulation of miR-34b and c correlated with presence of *p53* mutation, miR-34a is frequently downregulated even in cancers with wild-type p53. These observations indicate that miR-34 may have p53 independent role in cancer pathogenesis. We have also observed inverse correlation between expression of miR-34a and its predicted target MET expression in human EOC specimens, suggesting that reduced miR-34a expression is responsible for increased MET expression. To confirm this possibility we have reconstituted p53 deficient cells, whose miR-34 family expression was significantly reduced, with miR-34 precursor molecule and shown that such reconstitution reduces MET expression. Indicating importance of miR-34 of cancer suppression, reduction of MET expression significantly diminishes cell migration and invasion.

These studies, together with literature reports of frequent *p53* mutations, high level of MET expression and their association of these alterations with poor prognosis in EOC, have indicated that p53/miR-34/MET pathway plays a crucial role in epithelial ovarian carcinogenesis. To further explore p53/miR-34/MET regulation in EOC, we have inactivated *p53* in OSE cells and studied MET expression, cell motility and invasion by using primary OSE cells from $p53^{loxP/loxP}$ mice. Consistent with previous observations, MET expression is increased soon after *p53* inactivation *in vivo,* as well as in OSE cell culture. Importantly, increased motility and invasion resulting from *p53* inactivation is abrogated by *Met* inactivation, indicating that *p53* controls cell motility and invasion dependents on regulation of MET expression. By using primary OSE cells deficient for all miR-34 family (see below) we have directly shown that in addition to earlier observations of miR-34-mediatred MET regulation by p53, it also regulates MET by miR-34 independent mechanisms. We have determined that in the latter variant p53 is able to repress *MET* promoter activity through transcriptional factor Sp1, and shown that p53 may inhibit Sp1 DNA binding through protein-protein interactions. Notably we have shown that several p53 proteins containing various DNA binding domain mutations retain *MET* promoter suppression, thereby resulting in somewhat lower expression levels of MET as compared to cells null for p53. This finding may provide mechanistic explanation to reports of poorer prognosis of patients with p53 null EOC, as compared to those expressing mutant p53 (Rose et al., 2003; Shahin et al., 2000; Sood et al., 1999).

In order to study the precise role of miR-34 as a potential tumor suppressor *in vivo,* we have generated *mir-34a* conditional knock-out mice, and obtained conventional and conditional *mir-34* family knock-out mice in a collaboration with David Corney (our laboratory) and Dr. Heiko Hermeking (Ludwig-Maximilians-University Munich). We have demonstrated that mice lacking all *mir-34* genes are alive, similar to p53 null mice. However, our initial pathological evaluation indicates that *mir-34* null mice have pathological alterations distinct from those associated with

p53 deficiency. Unlike early onset lymphomas typical for p53 null mice, *mir-34a*$^{-/-}$*mir-34b/c*$^{-/-}$ mice have hypoplasia of the spleen, accelerated aplasia in the thymus, focal hyperplasia of the Purkinje cells and ulcerations in the forestomach. We have also determined that unlike p53 *mir-34* null cells do not accelerate iPS cell reprogramming and may have different response to DNA damage by IR. These results provide essential basis for further elucidation of miR-34 biological functions in normal development and carcinogenesis.

5.2 Future directions

5.2.1 miR-34 and therapeutics in clinically relevant settings

p53 gene delivery by adenovirus has failed due to multiple genetic and epigenetic dysregulations in cancer patients, negative cross-talk between delivered wild-type p53 and dominant negative form of mutant p53, and interaction with p63/p73 (Zeimet and Marth, 2003). Our studies indicate that other means such as miR-34 delivery could be appealing alternatives to p53 based gene therapy.

In Chapter 2, we showed frequent downregulation of miR-34 family in ovarian cancer patients. In addition, reconstitution of miR-34 expression could reduce cancer cell proliferation, migration and invasion, suggesting a possibility of miR-34 delivery as a therapeutic agent in cancers, such as EOC with reduced miR-34 expression. Although downregulation of miR-34b/c was highly associated with *p53* mutational status, we found that cancers harboring wild-type p53 frequently also had reduced miR-34a expression as compared to normal human OSE cells. Moreover, regardless of p53 status, some patients had methylation of *mir-34* promoters and gene copy number variations, suggesting that miR-34 biological functions regulated

independently of p53 are also important for cancer pathogenesis. Consistent with this possibility, miR-34 regulation by other transcription factors, such as c-Myc, ELK1 and C/EBPα, has been reported (Chang et al., 2008; Christoffersen et al., 2010; Pulikkan et al., 2010). Interestingly, in the case of pleural mesothelioma, characterized by rare *p53* mutation, CpG island methylation of *miR-34* promoter region is frequent and associated with reduced expression (Kubo et al., 2011). Taken together, recent data from various types of cancers suggest that miR-34 can be regulated by p53-independent mechanism and downregulation of miR-34 in malignancies frequently occurs regardless of *p53* mutational status. Despite sequence similarity and several shared targets of miR-34 family, individual miR-34 family may have different targets and effects when expressed (Cole et al., 2008). Therefore, it is reasonable to further test the effect of individual miR-34 delivery for cancer therapy in mouse models.

In order to explore therapeutic potential of miR-34 in animal models, it is required to have accurate animal models and experimental system to address this issue. Previously our laboratory have reported a model of EOC peritoneal dissemination initiated by intraperitoneal injection of neoplastic OSE cells (Williams et al., 2010). This system has several advantages over other experimental disease progression models. First, primary OSE cells derived from genetically modified mice are far better genetically defined, unlike human cancer cell lines, which usually have numerous passenger mutations. Second, unlike human cancer cell lines xenografted into immunocompromised mice, neoplastic OSE cells can be injected into immunocompetent syngeneic mice, allowing tumor development in physiologically adequate microenvironments. Using newly developed *mir-34* knock-out mice in conjunction with other genetic alterations, we are able to generate neoplastic OSE cells with either intact miR-34 expression or miR-34 deficiency. Furthermore, lentivirally infected GFP expression system enables us to monitor tumor growth and regression in live animals. Since introduction of lentiviral vectors usually results in the heterogeneity in transgene expression levels among cells

due to differences in the position of their integration, we will use a modified pINDUCER lentiviral vector (Figure 5.1) (Meerbrey et al., 2011). This vector will allow controlled miRNA-expression by tetracycline/doxycycline controlled reverse transactivator (rtTA) in parallel with expression of two reporter genes. Thus both integrated vector and miRNA induction can be tracked by different fluorescences. Along with lentiviral system, in collaboration with Dr. Moonsoo Jin lab, we are developing nanoparticle-mediated miR-34 delivery in ovarian cancer models. In this way, we can systemically deliver miRNAs and evaluate the effect of miR-34 delivery and side effects. Therefore, taking advantages of newly developed *mir-34* conditional knock-out mice, new generation of inducible lentivirus system and nanotechnology to deliver miRNAs, we will be able to examine the therapeutic potential of miR-34 in EOC.

5.2.2 p53/miR-34/Met pathway in early carcinogenesis

It is obvious that MET regulation is important in carcinogenesis, since its overexpression is frequently associated with poor prognosis in several different types of cancers including ovarian carcinoma (Masuya et al., 2004; Sawada et al., 2007; Tolgay Ocal et al., 2003). Various approaches to regulate MET by antibody, siRNA or small molecules have been in clinical trials (Comoglio et al., 2008). Importance of MET regulation could be further supported by our finding that p53 has dual regulation of MET via miR-34-dependent and -independent pathways. It has been proposed that this type of redundancy, also known as feedforwardloop regulation, ensures prevention of leaky expression (Hornstein and Shomron, 2006; Stark et al., 2005; Tsang et al., 2007). Therefore, by using feedforward loop regulation of MET, p53 is able to tightly regulate MET expression both on transcriptional and post-transcriptional levels. Identification of MET as an important target of p53 in EOC has two significant implications.

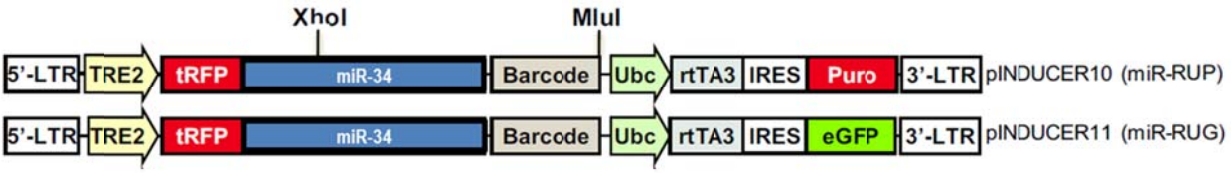

Figure 5.1 The pINDUCER lentiviral vector for inducible miR-34 system. Modified from Meerbrey et al. (Meerbrey et al., 2011)

First, our studies show that metastatic traits such as cell motility and invasion mediated by MET could be acquired during early stages of carcinogenesis initiated by *p53* mutations. These findings support a recent shift in paradigm of sequential cancer progression. According to the classical carcinogenesis model, metastatic traits have been thought to be acquired at late stages of carcinogenesis (Gupta and Massague, 2006). However growing number of evidences point to a possibility that some neoplastic cells may have metastatic ability already during the early stages of carcinogenesis (Eyles et al., 2010; Podsypanina et al., 2008; Schardt et al., 2005; Weinberg, 2008). This might explain the reason for fast progression and difficulties to identify precursor lesion of high grade serous type EOC.

Second, it has been far less appreciated that wild-type p53 is a critical regulator of cell motility and invasion, albeit Rho family of small GTPase has been implicated in cell motility as a p53 function (Gadea et al., 2007; Gadea et al., 2002; Gadea et al., 2004). Rather, gain-of-function mutation of *p53* has been considered to be associated with metastatic traits (Brosh and Rotter, 2009; Olive et al., 2004; Oren and Rotter, 2010). Our finding that wild-type p53 controls cell motility and invasion mainly through MET sheds light on these less appreciated functions and may facilitate use of p53 molecular signatures for disease prognosis. Prediction of patient prognosis based on *p53* mutational status has been controversial (Soussi and Beroud, 2001). However a number of evidences consistently indicate that *p53* null mutations are associated with the worst course of cancer progression as compared to mutations resulting in expression of mutant p53 in lung, breast and ovarian carcinoma (Hashimoto et al., 1999; Lai et al., 2004; Olivier et al., 2006; Pavel et al., 2008; Rose et al., 2003; Shahin et al., 2000; Sood et al., 1999). In Chapter 3, our studies have shown molecular mechanism which may explain differential prognosis based on *p53* mutational status (Figure 5.2).

Since DNA binding domain mutant p53 still retains partial suppression of *MET* promoter but loses transactivation of miR-34, MET expression would be less affected, likely leading to

more limited invasive and metastatic properties of neoplastic cells, as compared to those in p53 null cancers (Figure 5.2). Therefore, it would be of interest to determine if affecting individual components of feedforward loop regulation of MET will result in differential MET expression and prognosis in human ovarian cancer carrying different types of *p53* mutations. In addition, it would be interesting to study if this feedforward loop regulation is also found in other types of normal and neoplastic cells. Our preliminary studies on colon and lung cancer cell lines suggest that such regulation of MET does exist in other types of cancer (Chapter 3).

With regard to miR-34-independent mechanism, the involvement of Sp1 may lead to a novel approach for regulation of MET expression. Both mithramycin A treatment to inhibit Sp1 DNA binding and Sp1 knockdown by siRNA generate similar effects on MET expression and promoter activity (Papineni et al., 2009; Verras et al., 2007; Zhang et al., 2003). Recently, tolfenamic acid, a small molecule of Sp1 inhibitor, which is able to induce Sp1 family protein degradation has been identified (Colon et al., 2011). Expectedly, this inhibitor downregulates MET expression in cancer cells, including ovarian cancer cells (Basha et al., 2011; Papineni et al., 2009). Interestingly, we have observed that some cancers harboring p53 also highly express MET. In the case of ovarian cancer cell line OVCA433 the enrichment of Sp1 on the proximal region of *MET* promoter likely results in MET overexpression (our unpublished data). This data suggests that there is an alternative pathway to increase MET expression by recruitment of Sp1 in the promoter region. Consistent with this possibility, we observed continuous increase in MET expression after *p53* inactivation with the highest expression of MET in late passage cells (Chapter 3). Detailed understanding of p53/miR-34/MET pathway and the involvement of Sp1 in miR-34-independent regulation of MET should pave the road toward alternative therapeutic approaches.

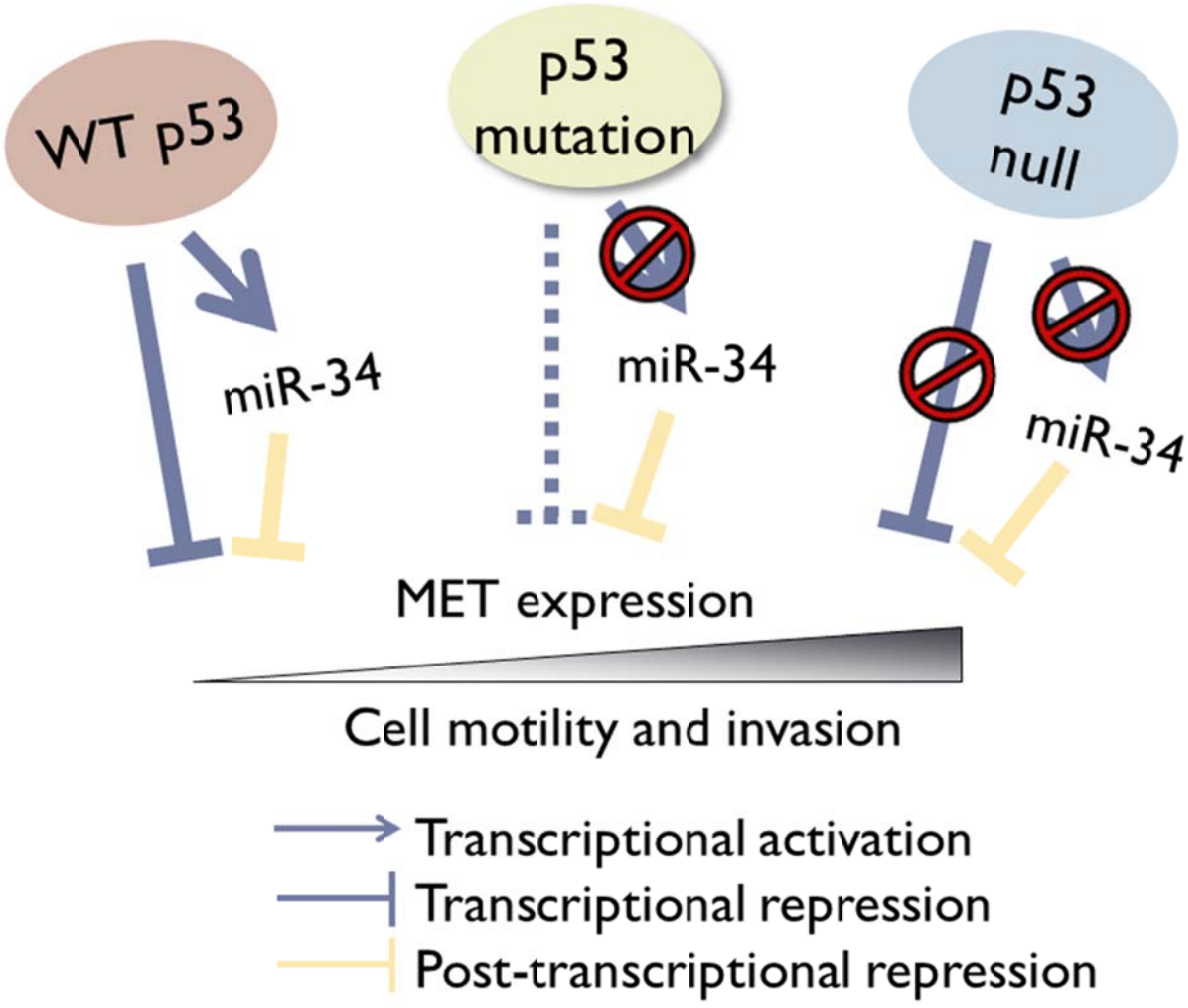

Figure 5.2 Proposed model of differential effects of *p53* mutations on feedforward loop regulation of MET.

5.2.3 The role of miR-34 in development, carcinogenesis and DNA damage response

It is not completely surprising that *mir-34a*$^{-/-}$*mir-34b/c*$^{-/-}$ mice are grossly normal and did not show any embryonic lethality, since *p53*$^{-/-}$ mice are also grossly normal and lack any observable gross defects (Donehower et al., 1992). At the same time, our initial observations indicate that miR-34 family may have a different role in development and/or carcinogenesis. In our preliminary data, one of the obvious observations was an abnormality in hematopoietic system, thymus and spleen. Thymus from 9 weeks old *mir-34* deficient mice undergoes accelerated aplasia, and spleen showed hypoplasia and/or aplasia of white pulp. In accordance with our observations, miR-34a has been shown to be involved in B and T cell development (Rao et al., 2010; Stadanlick et al., 2011). In particular, ectopic expression of miR-34a blocked pro-B-cell-to-pre-B-cell transition in B cells and induced arrest of T cells. It is reasonable to hypothesize that elevation of a certain miR-34 target proteins may result in this phenotype. Rao et al. further demonstrated that blocked B cell development by miR-34a might be due to decreased level of FoxP1, a novel target of miR-34a (Rao et al., 2010). Therefore, further examination on FoxP1 expression and B cells development may explain an observed splenic phenotype of in *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$mice. Accelerated thymic aplasia has been observed in TNF and IL-10 transgenic mice (Liepinsh et al., 2009; Rouleau et al., 1999). It is tempting to speculate that miR-34 family has certain unidentified target genes among cytokine-related genes, whose gene expression were increased in the thymus of *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ mice. Therefore, identification of cytokine-related genes targeted by miR-34 could be one of areas of future interest.

Based on tissue distribution of miR-34a and miR-34b/c, miR-34b/c is highly enriched in testis and has been implicated in spermatogenesis (Bouhallier et al., 2010), while miR-34a is ubiquitously expressed throughout tissues (Diez-Roux et al., 2011). However, *mir-34b/c*$^{-/-}$ mice

showed no gross abnormalities in testis and fertility, suggesting that miR-34a might compensate for loss of miR-34b/c in testis. Consistent with this possibility, our preliminary results showed lower fertility in *mir-34a*$^{-/-}$*mir-34b/c*$^{-/-}$ mice, albeit it is too early to make any conclusion. This observation raises a question what is the precise role of miR-34 in spermatogenesis.

It is also tempting to speculate its potential role in stem and/or progenitor cells. According to literature reports (Christoffersen et al., 2010; Kong et al., 2008; Lujambio et al., 2008) and our unpublished data from our laboratory (Chang-il Hwang, David Corney, Kirsten Elzer, and Alexander Nikitin) miR-34 has been predicted and experimentally validated to target Klf-4 and c-Myc, which are required transcription factors for iPS cell reprogramming. In addition, other studies indicate adult stem cells of some tissues may have higher expression of miR-34 family (David Corney, Samantha Palmaccio, Chang-Il Hwang, and Alexander Nikitin, unpublished). In agreement with these observations, Notch1, Notch2 and Jag1 have been shown as targets of miR-34 (Lewis et al., 2003; Pang et al., 2010). Exhausting of stem/progenitor cell pool could be a one of the reason for thymic and spleen hypoplasia observed in mice null for all *mir-34* genes. Therefore, the effect of *mir-34* inactivation in diverse tissue stem cells is worth to be explored.

To evaluate role of *mir-34* in carcinogenesis we plan to cross *mir-34a*$^{loxP/loxP}$*mir-34b/c*$^{loxP/loxP}$ mice with tissue-specific *Cre* transgenic mice, such as PB4-*Cre* to induce gene inactivation in prostate epithelium (Wu et al., 2001), and MMTV-*Cre* to induce gene inactivation in mammary epithelium (Cheng et al., 2010). To study ovarian carcinogenesis, intrabursal administration of Ad-*Cre* allows inactivation of *mir-34* in OSE (Flesken-Nikitin et al., 2003). Since *mir-34a*$^{-/-}$*mir-34b/c*$^{-/-}$ mice did not develop spontaneous tumors by 6 months after birth unlike p53 deficient mice (Donehower et al., 1992), we will also introduce other genetic alterations such as *p53* and/or *Rb mutations*, and expression of mutated *K-Ras* to see if miR-34 cooperates with other tumor suppressors or oncogenes.

Given survival difference of *mir-34b/c*$^{-/-}$ in 15 Gy of whole body irradiation, it seems to be obvious that miR-34 family plays an important role in gastrointestinal tract syndrome induced by ionizing radiation (IR). Comprehensive analysis of the effect of IR on intestinal epithelium including apoptosis, proliferation and senescence should be carried out. Although we were not able to see survival difference in 10 Gy of IR for hematopoietic syndrome, it is worth to explore the effect of 10 Gy whole body irradiation on *mir-34a*$^{-/-}$ and *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ mice. Since miR-34a has been connected to B and T cell development as described above and our results suggest that miR-34 appears to play a role in hematopoietic system development, we may be able to see some differences in hematopoietic syndrome induced by 10 Gy of IR. In addition, since thymic accelerated aplasia has been observed in 9 weeks old*mir-34a*$^{-/-}$*mir-34b/c*$^{-/-}$ mice but not at 3 weeks of age, DNA damage response in different age of *mir-34a*$^{-/-}$*mir-34b/c*$^{-/-}$ mice will be subjected to our next studies.

In conclusion, we have explored the role of p53/miR-34/MET pathway in ovarian cancer specimens, demonstrating that frequent downregulation of miR-34 is mainly associated with *p53* mutation and MET overexpression. Our project to utilize primary OSE cells from genetically modified mice as an early model of ovarian carcinogenesis allowed us to identify p53 as a master regulator of cell motility and invasion, and to show that MET signaling is essential component of such regulation. Also we have shown that p53 is able to regulate MET via miR-34-dependent and miR-34-independent mechanisms. In order to directly prove a hypothesis that miR-34 is a tumor suppressor, we have generated *mir-34a* conditional knock-out mice and are in the process of characterization of *mir-34a*$^{-/-}$ *mir-34b/c*$^{-/-}$ mice. Characterization of the effect of *mir-34* inactivation in diverse tissues will allow us to evaluate the precise role of miR-34 in development, DNA damage response and carcinogenesis.

5.3 References

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